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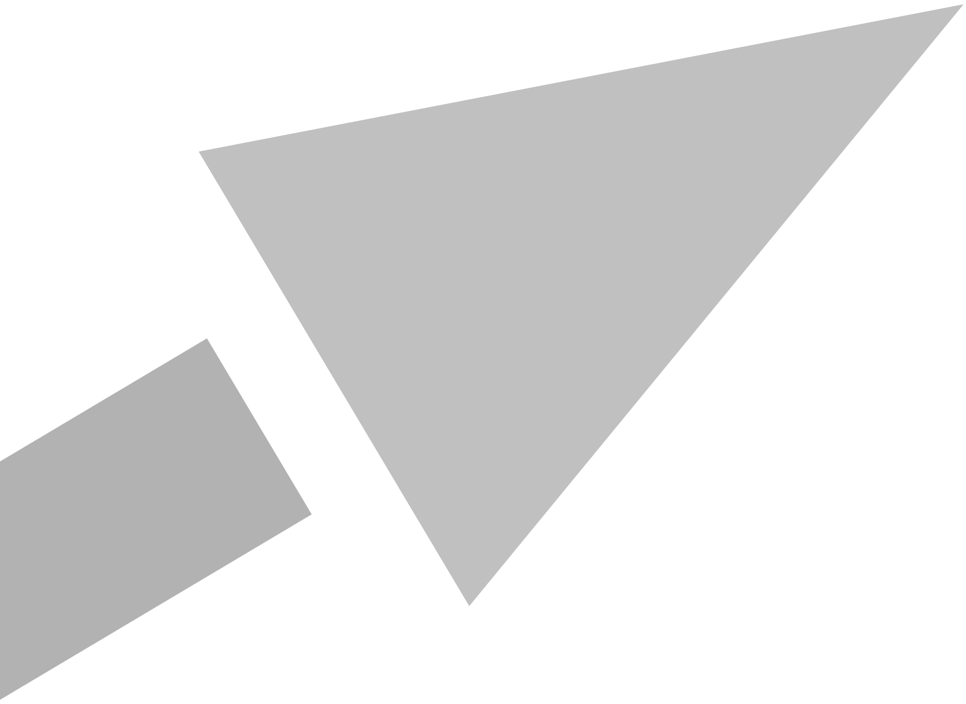

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Contents

SUMMARY

The aims of this report are to review the state-of-the-science for the testing of undissolved chemicals, from the nano to the macro scale in aquatic toxicity tests and to identify test methods applicable to the testing of such substances. In reviewing aquatic toxicity data that have been published for particulates in the peer-reviewed literature, such as nanomaterials (NMs) and microplastic (MP), as well as for poorly soluble liquids, several fundamental challenges emerge. Specifically, factors related to controlling uniform dispersions, variability in organism physiological and behavioural traits that might influence uptake and bioaccumulation, and lack of standardisation with respect to duration of exposure, consistency in exposure metric, and reporting of apical effect endpoints, are representative of information that is generally inconsistent between studies with limited attempts to address these issues. The lack of standardised approaches thus complicates the communication of potential environmental risks associated with exposure to particulates.

A general trend in the testing of particulates has been to simply adopt standardised OECD aquatic toxicity tests, which have been developed for soluble chemicals, and to expose organisms to particles, such as NMs and MP, up to an amount that results in an observed adverse effect (OAE). It is argued that an OAE is the sum of effects due to an intrinsic toxicity (E_{iT}) associated with a substance and direct and indirect effects that might result because of a physical interaction (E_{Pint}) between the substance and organism (i.e. OAE = $\Sigma E_{iT} + \Sigma E_{Pint}$). For soluble chemicals tested in standardised OECD aquatic toxicity tests, test methods have been developed to specifically eliminate the potential of E_{Pint} to influence the OAE. This is achieved by ensuring the concentration of the chemical in the test system does not exceed the solubility limit for the chemical. The tests are therefore designed to characterise and quantify the E_{iT} of a chemical substance, information which can then be used to more accurately characterise the potential environmental risks. The approach for testing particles, however, is characterised by studies that do not define a maximum exposure limit for the particle concentration, which results in organisms showing direct and indirect effects that are largely a consequence of E_{Pint}. Such observations are thus problematic, in that they do not lend themselves to standard concentration dose-response relationships, and therefore cannot be used within a classical risk assessment framework.

To advance the scientific value of aquatic toxicity testing for particles a main observation in this report relates to an urgent need to strengthen our quantitative and mechanistic understanding of the relationship between the intrinsic physicochemical properties of particles and OAEs, in helping to screen and prioritise the development and application of aquatic toxicity testing methods. Application of OAEs within risk assessment, however, will also require advances in our understanding between intrinsic and extrinsic properties that influence exposure, both within standard test methods as well as under environmentally relevant conditions. This report thus characterises the key challenges and limitations associated with developing guidance needed by regulators in applying data obtained from aquatic toxicity studies within a risk assessment framework that specifically addresses exposure to particles released to the environment. Addressing these challenges will require multi-stakeholder engagement, where there is a need to develop consensus on how to best assess the risks associated with exposure to particles that originate from commercial activities. A key recommendation from this report is to thus enable and support mechanisms that bring together all key stakeholders, such as through the organisation of multi-stakeholder workshops that attempt to address specific challenges. The following summarises several key areas that would benefit from constructive multi-stakeholder input:

- Development of clear, common definitions for particle categorisation and exposure metrics.
- Consensus on reporting requirements aligned to both the exposure stressor and exposure event, including particle intrinsic and extrinsic properties and test system extrinsic properties most likely to influence aggregation, agglomeration, sedimentation, dissolution, etc.
- Analytical support to fully characterise material being tested in addition to the inclusion of chemical leaching controls (monomers, chemical additives, etc.) as deemed appropriate.
- Development of protocols for creating and maintaining dispersions, sample preparation, and analytical methods to minimise test artefacts and strengthen reproducibility and interpretability
- Standardised methods to assess environmental transformation processes, such as assessing OAE associated with exposure to environmentally relevant aged MP or NMs.
- Development and use of standard reference materials for method validation and test control.
- Research aligned to identifying and prioritising environmentally relevant exposure scenarios and/or benchmarks.
- Clarification and consensus of environmental protection goals associated with potential releases of particles.
- Identification of sentinel test species based on species sensitivity distributions that build on mechanistic understanding of physiological and behavioural traits.
- Consensus regarding appropriate effect endpoint(s).
- Development of research that elucidates mechanistic differences between E_{iT} and E_{Pint}.

Finally, it should be noted that the observations and recommendations presented in this report are consistent with several other reviews and summaries that have recently been published in relation to assessing the risks of NMs and MP. For instance, an important observation relates to the potential opportunities to adopt learnings from one area of research to another, such as between the advances in the testing of NMs, which might facilitate the adoption of standardised methods targeted for MP. Furthermore, the inclusion of poorly soluble liquids within the discussion of this report provides another area of science where learnings associated with preparing and controlling dispersions to represent environmentally relevant scenarios may also prove useful in addressing the testing of NMs and MP. Thus, it is anticipated that discussions and research that helps to address the challenges identified in this report, relevant to any chemical substance for which E_{Pint} likely dominates an OAE in an aquatic toxicity test, would not only benefit our overall ability to address the concerns that have recently emerged in relation to NMs and MP but would also help to strengthen future innovation associated with ‘safer-by-design’ strategies.

1. INTRODUCTION

In 1996 ECETOC published Monograph No. 26 (ECETOC, 1996), which reviewed the difficulties of sparingly soluble substances in aquatic toxicity assays. This work helped establish the current paradigm in aquatic environmental risk assessment, accepted by authorities globally, that the dissolved molecule represents the most relevant (and worst case) exposure condition for aquatic toxicity testing, and that testing above the solubility limit does not generally help to inform environmental risk. The increasing focus on NMs and MP in the environment over the last decade is challenging this paradigm, and has inspired debate regarding the adequacy of existing aquatic testing frameworks for chemicals that have potential for emission and transmission to the aquatic environment in varied undissolved/particulate states (Skjolding et al., 2016; Hansen et al., 2017; Koelmans et al., 2017; Steinhäuser and Sayre, 2017; Oomen et al., 2018). Much of the published research that has investigated the potential ecotoxicity of nanomaterials has employed procedures involving exposure to these substances above the solubility limit and in the presence of undissolved substance (Hjorth et al., 2017). Relatively little attention is given to defining the physical states of particulates or undissolved substance associated with known or expected exposure pathways, or the distinction between intrinsic toxicity and physical effects associated with those relevant physical states (Hjorth et al., 2017). The latter is often a fundamental requirement in regulatory aquatic toxicology studies. Indeed, aquatic toxicity tests at concentrations exceeding the aqueous solubility of a chemical are often considered unacceptable by regulatory authorities for risk assessment purposes (ECETOC, 1996; ECHA, 2011).

The need for guidance on when and how to test chemical particulates in aquatic toxicity tests has re-emerged because of the apparent bioavailability of some nanomaterials in their undissolved state. Several current activities at the OECD are focused on developing guidance for aquatic hazard and bioaccumulation testing of nanomaterials (OECD, 2010; OECD, 2011; OECD, 2012; OECD, 2014; OECD, 2015a; OECD, 2017a; OECD, 2017b; Oomen et al., 2018). However, there is no consensus in the scientific community that particulates below a certain size cut off (e.g. 100 nm) pose any unique hazard or risk to aquatic organisms (Sayre et al., 2017; Cana, 2018). Moreover, there is no single regulatory definition of a nanomaterial, distinguishing it from a 'conventional' particulate chemical, which is globally accepted (Sayre et al., 2017). In Europe, the European Commission has issued a risk-neutral recommendation of definition of a nanomaterial (EC, 2011a), of such a wide scope that it could be interpreted to encompass most solid particulate chemicals, regardless of manufacturing intent. If the OECD adopts new test strategies and recommendations for aquatic tests with nanomaterials, there is the potential that existing studies for sparingly soluble chemicals, deemed nanomaterials by some subsequently applied definition, may be considered insufficient for characterising exposure and risk; and new studies following nanomaterial testing recommendations would be requested (Sayre et al., 2017; Cana, 2018). Fundamentally, there is a need to examine circumstances when studies conducted under existing standardised testing procedures are appropriate for use in risk assessment and to derive clear guidance for when specific tests, which include undissolved test chemical in various physical states, may be warranted, along with a scientifically sound approach to link measured effects to risk assessment goals (Expert panel on nanotechnolgy, 2008; Skjolding et al., 2016; Hansen et al., 2017; Steinhäuser and Sayre, 2017).

Aquatic toxicity tests and risk assessment frameworks were designed based on existing infrastructure and engineering controls, which minimise aquatic exposures to chemicals in undissolved states. Thus, for most conventional substances and associated life cycle stages, the presumed exposure modes involve contact of aquatic organisms with a fully dissolved concentration of the toxicant (ECHA, 2011). The terminology currently used to describe aquatic hazard and risk largely reflects the assumption that the chemical partitions into biota to achieve an equilibrium between the external dissolved concentration and an internal dose (bioconcentration), which at a critical level manifests as toxicity (McCarty and Mackay, 1993; Escher and Hermens, 2002; Escher and Hermens, 2004). In practice, toxic effects seen in aquatic tests are best described in relation to the dissolved test chemical concentration. These are concepts with foundations in fundamental chemical principles. The current terminology and metrics used to describe hazard and risk were not intended to characterise the physical exposure modes and physical injury from undissolved particulates. For example, dispersed particulates do not achieve a state of equilibrium partitioning, so injury from particulates is generally poorly described by applying toxicological concentration-response models (Skjolding et al., 2014; Hu et al., 2016a; Skjolding et al., 2016; Hansen et al., 2017). Language and procedures may need to evolve to better reflect the dynamic nature of particulate exposure and to adequately describe their associated risks (Palmqvist et al., 2015).

Industry and regulators have shared responsibility for assuring that appropriate exposure, hazard, and risk assessments are made for manufactured substances considering both the physicochemical properties and test organism life cycle stages. In this light, the ECETOC Scientific Committee commissioned a Task Force charged with the following **Terms of Reference**:

- To build upon and update the guidance provided in ECETOC Monograph No. 26 (1996) by critically re-examining the relevance of undissolved chemicals, from the nano to the macro scale, in aquatic toxicity tests with emphasis on uptake and bioaccumulation.
- To identify circumstances where current aquatic hazard testing approaches are acceptable and make recommendations where a different approach may be warranted for sparingly soluble particulates, including nanomaterials.
- To develop guidance which assists distinguishing intrinsic toxicity from physical effects, characterising physical effects, and identifying circumstances where physical effects may contribute to population relevant risk.

The discussion presented in this report thus aims to characterise the key challenges and limitations associated with developing guidance needed by regulators in evaluating aquatic toxicity studies conducted with chemicals in an undissolved state, and to relate any observed adverse effects to relevant modes of aquatic exposure. It is the intent of this report to provide an instrument that can be used to help guide critical discussions needed towards establishing regulatory guidance that can effectively distinguish hazard contribution from intrinsic toxicity and physical injury/effects.

It should be noted, that the emphasis of the discussion presented in the report pertains to the aquatic toxicity testing of manufactured chemicals and materials. Several related areas have been excluded from the report as these topics are beyond the scope of the Terms of Reference defined for the Task Force:

- The results pertaining to terrestrial and sediment toxicity testing.
- The role of particulates as vectors of transport for other environmental pollutants.
- The environmental fate and effects associated with the formation of degradation products, such as secondary MP formed following the release of manufactured primary MP or macroplastic.

Nonetheless, many of the observations made in relation to the challenges and limitations identified for aquatic test systems are likely to provide valuable insight to those topics identified as being beyond the scope of this report.

1.1 Regulatory drivers

In assessing the environmental and human risks associated with exposure of particles present in consumer products or which might be encountered in the environment, there are several key stakeholders that can influence the development and implementation of regulatory drivers. The key stakeholders include industry and academic interests with respect to innovation and development of new materials to benefit society, which also need to be balanced with ensuring both the human and environmental safety regarding the use of those materials. Scientific research and its publication in the peer-reviewed literature by academics often develops from data generated from non-standard GLP experiments, which test a myriad of hypotheses. Initial observations from these tests can be used to further advance scientific understanding. Both industry and regulators monitor ongoing advances in academic research, whereas the general-public typically become aware of data that indicates an emerging concern associated with the use and exposure to materials used in commerce and found in the environment often through non-governmental organisations (NGOs) via the news media. As a general observation, once an issue of emerging concern manifests itself within the public domain, the general-public can have a strong influence over the direction and speed of adoption of new legislative instruments (Halden, 2015). Consequently, early engagement with all stakeholders is seen as an important mechanism early in the process to determine the major factors to be considered, the decision-making context, and the timeline and depth needed to facilitate the constructive development of science to address immediate concerns (NRC, 2009). Figure 1 provides a schematic illustration of the key stakeholders and the challenges needed to metaphorically balance societal benefits that accompany technological innovations with human and environmental safety. The primary goal of all stakeholders is to ensure human and environmental safety, although the mechanisms driving the process for each may differ and thus influence the efficiency of the overall process.

Figure 1: Schematic illustration of key stakeholders, their relative roles and drivers, and relationships between them that enable the adoption of instruments to ensure the human and environmental safety of materials and chemicals used in commerce.

From a European regulatory perspective, the assessment of risks is overseen by various independent bodies, which give scientific advice to decision makers at the European Commission (Heyvaert, 2008). In addition to the three Scientific Committees managed by DG SANTE (the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR)), the EU Risk Assessment system also includes the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Chemicals Agency (ECHA), the European Centre for Disease Control and Prevention (ECDC), the European Environment Agency (EEA), and the Scientific Committee on Occupational Exposure Limits (SCOEL), managed by DG Employment. The approach taken within Europe, however, is not inconsistent with other national approaches, where risk assessment is addressed by various regulatory bodies and legislative instruments (Expert panel on nanotechnolgy, 2008; OECD, 2015b; OECD, 2017a).

The role that the various stakeholders play in influencing the direction of regulatory instruments aligned to assessing the hazard of particles of a wide range of materials, either natural or synthetic in origin, have differences and similarities (Halden, 2015; Syberg et al., 2015; Hüffer et al., 2017). In this section, we introduce some of the political issues and scientific challenges that accompany the development and adoption of regulatory instruments, using engineered nanomaterials (NMs) and MP as representative examples. Key differences and similarities are thus summarised, with an emphasis on identifying those potential learnings that have been made in strengthening the development and implementation of standardised approaches for assessing adverse effects associated with exposure to particulates, and which might be applied to any chemical or material where a physical effect, which differs from an intrinsic toxicity of the material, is observed.

1.1.1 Engineered nanomaterials

Although nanotechnologies have been considered as emerging technologies for more than a decade, they still face rapidly evolving regulatory frameworks. One of the main challenges is the absence of a single, worldwide, and commonly agreed definition associated with the terms engineered nanomaterials, nanoparticle, nanoform or nanostructured material (SCENIHR, 2010; Sayre et al., 2017; Cana, 2018). The past 5 years, however, has seen intensive development of regulatory frameworks. Among them, the European Recommendation of a definition for NMs (EC, 2011a) or the European Cosmetics Regulation definition (EC, 2009), together with the emergence of new concepts and definitions (e.g., the nanoform definition which is currently developed and possibly included in the amended REACH Annexes for nanomaterials (ECHA, 2017a; Cana, 2018)) or the release of ISO documents detailing a comprehensive vocabulary related to nano-objects, nanofibers, nanoplates, and the safety evaluation associated to these concepts (OECD, 2015b). According to the European recommendation (EC, 2011a), the definition of a 'nanomaterial' is: a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm[1]. In specific cases, and where warranted by concerns for the environment, health, safety or competitiveness, the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%. By interpreting the above, fullerenes, graphene flakes and single-wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as NMs. This recommendation, however, is not the only proposed definition currently existing. Since 2007 (U.S. Environmental Protection Agency (EPA), 2007), and updated more recently in 2014 (NRC, 2014), the US Environmental Protection Agency has proposed the following key criteria in a proposal for a definition:

- The NMs should be a solid at 25°C and atmospheric pressure.
- A particle size between 1 and 100 nm in at least 1 dimension should have been measured.
- The material exhibits unique and novel properties because of its size.
- The material is engineered at the nanoscale.
- The distribution of particles should include more than 10% by weight for particles of size less than 100 nm.

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[1] Some controversy exists with the EC definition, which includes the lack of manufacturing intent and no consideration of mass. With the EC definition you could have a scenario whereby one manufactures 100 bowling ball size objects, however, amongst those macro-sized materials are 100 nano-sized balls, then the bowling ball size objects would thus be classified as nanomaterial. Please note that this issue is addressed in the USEPA definition.

Some EU regulations have set NM definitions, each with a slightly different scope:

- The Cosmetics regulation (1223/2009) (EC, 2009).
  'NM' means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm.
- The Biocides regulation (528/2012) (EC, 2012).
  'NM' means a natural or manufactured active substance or nonactive substance containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm.
- The Food Information to Consumers regulation (1169/2011) (EC, 2011b).
  'Engineered nanomaterial' means any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale.

It is likely that newer definitions of NMs may emerge not only in the EU or US, but in other parts of the world as well, based on highly specific applications (CIEL, 2014).

REACH and Nanomaterials

REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) is the European Union regulation, dated December 18th 2006, addressing the production and use of chemical substances (EC, 2006). Guidance and documents issued in Europe reconfirm that the current regulatory framework is applicable to NMs (ECHA, 2011). Recently, a specific guidance was issued for NM (ECHA, 2017b) and a discussion is ongoing on the possible revision of the annexes of REACH for the specific data requirements for NMs, even if it was first acknowledged in 2007 by SCENHIR that NMs should not be treated distinctively in evaluation programs (SCENIHR, 2007). A public consultation on the possible amendment to the REACH annexes was launched during the finalisation of this ECETOC report and the final outcome of this consultation are not known at the time of preparing this publication. Some additional information requirements may be defined in the near future for NMs. For instance, a more elaborate physicochemical characterisation to support grouping approaches and read-across is foreseen. The NANoREG project, funded by the Framework Programme 7 of the European Commission (www.nanoreg.eu), combined researchers from academia, national authorities, and industry, not restricted to Europe, evaluated the appropriateness of the repertoire of OECD guidelines to a collection of NMs differing in terms of chemical composition, shape, and surface area. Some tests for aquatic toxicity or the Ames test in particular, have already shown limits while evaluating NMs (Handy et al., 2012). The results of the NANoREG project were presented during a conference organised during 2016 jointly with the Prosafe project, with a conference white paper reporting on the evaluation of NMs being recently published (Gottardo et al., 2017).

Current National Context: Inventories

National mandatory inventories have entered into force across Europe, such as in France (French Decree 2012-232), in Denmark (Statutory Order No644 / 2014), in Norway (Regulations relating to the declaration of chemicals to the Product Register / 2013), and in Belgium (Royal Decree of 27 May 2014); a voluntary inventory is also under discussion in Italy. The scopes of these various inventories differ because some of them are focused on the substances and mixtures (like the notifications in France, Norway or Belgium) while others focus on final products (in Denmark; possibly in Belgium). The notification in Norway is relatively simple, consisting mainly of 'ticking-a-box' added to the typical chemical notification process in place. In France and Belgium, a web-based platform has been specifically designed as a tool to collect the required information. This information mainly pertains to:

- Identification of the substance (IUPAC name, CAS and EINECS numbers, commercial names).
- Administrative data related to production, formulation or distribution of the NM, a series of physicochemical characteristics (primary particle size, particle size distribution, agglomerate and aggregate sizes, shape, surface area, coating, impurities, zeta potential) the quantities handled with a minimal annual threshold of 100 grams driving the mandatory aspect of a notification.
- The uses of the products.
- The list of customers who would also have to declare their formulations if not consisting of final products (this information is kept confidential by the authorities).

The main objective of such inventories is to trace the presence of NMs in a national context and to better identify the actors in the domain. For these national regulations, the toxicological and ecotoxicological information can also be requested by the national authorities in case of further evaluation of a substance. These initiatives are not limited to Europe, for instance, similar activities have recently been launched by the US-EPA under the newly revised Toxic Substances Control Act (TSCA) (U.S. Environmental Protection Agency (EPA), 2018).

Perspectives

The current concepts addressing NMs evaluation are evolving to consider emerging scientific advances. Refinement of these concepts allows for the development of specific tools to monitor and evaluate the physicochemical specificities of NMs with regards to the test guidelines used to evaluate the hazards of nanomaterials. In a context of brisk growing science, a diversity of NMs differing by size, coating, or shape is also a driver for the emergence of newly developed screening methods allowing a categorisation of materials based on physicochemical characteristics together with a relevant minimal set of ecotoxicological data.

1.1.2 Microplastic

The occurrence of plastic debris particles in the marine environment and waterways represents an issue of increasing public concern. Plastic debris found in the environment has been categorised as being either macro (>5mm) or micro (<5mm) plastic and can originate from either primary or secondary sources. The main source of MP has been attributed to the breakdown of macro-sized pieces of plastic that have succumbed to weathering processes; primary sources are those originating from pellet loss from plastic manufacturing, abrasion of paint, textile fibres from washing, plastic beads used in sandblasting and as an exfoliant in cosmetic products (GESAMP, 2015; Jambeck et al., 2015; Lassen et al., 2015; UNEP, 2016; Verschoor et al., 2016; Boucher and Friot, 2017). It is further acknowledged that litter originating from land-based sources, such as from streets, parking lots, and storm drains represents a significant contribution of plastic to the marine environment. Jambeck et al. (2015), for instance estimate that 275 million metric tons of plastic waste was generated by populations within 50 km distance from the coast in 192 coastal countries world-wide in 2010, with 4.8 to 12.7 million tons entering the ocean, which is equivalent to 1.8% to 4.7% of the global production of plastic in 2010.

In response to public concerns, governments and regulators in several jurisdictions have introduced legislation to ban intentionally added or primary MP from entering the environment. To date, the legislation has largely targeted banning the use of plastic microbeads in rinse-off cosmetics used for exfoliating and cleansing purposes. National bans have, at the time of drafting this report, been implemented in the U.S. and Canada, with the UK, France, Belgium, Italy, Sweden, Finland, Australia, South Korea, and Taiwan developing similar legislative measures.

At the EU level, the Environment Council have adopted Council Conclusions on the Circular Economy Action Plan, calling for the European Commission to introduce a “proposal for a ban on micro-plastic particles in cosmetics and proposals to address other products generating marine litter as appropriate” (EC, 2018a). These conclusions provide an indication of the political and regulatory direction related to the use of primary MP in commerce, and demonstrate the strong political pressure for an EU ban on the use of MP that have the potential to enter the marine environment (EC, 2018c). In March 2018, ECHA requested a “Call for Evidence on possible restriction of microplastics” (https://echa.europa.eu/-/call-for-evidence-on-possible-restriction-of-microplastics), targeting intentional MP use across all industries.

The 7th Environment Action Programme calls for the development of an EU-wide “quantitative reduction headline target for marine litter, supported by source-based measures and taking into account marine strategies established by Member States”. The Circular Economy Package sets a target for reducing marine litter by 30% by 2020 for the ten most common types of litter found on beaches, as well as for fishing gear found at sea (EC, 2018a), and will address the following release of primary MP to the environment (several sources of secondary MP are also included, but not listed):

- MP intentionally added in the production of cosmetic products that are often washed directly into sewage systems.
- MP intentionally added into detergent products during manufacture that are often washed directly into sewage systems.
- Paint used to protect buildings which is worn/weathered away during use. MP (‘microspheres’) are also known to be intentionally added to some building paint products.
- The raw material used in the production of plastic products which can be spilled and lost during manufacture and transportation.
- MP (small, usually black particles originating from re-cycling of rubber tyres) used on the surface of an artificial sports pitch to provide shock absorption and prevent injury that can be transported away from the pitch by players and adverse weather.

- Plastic used in place of sand blasting as an abrasive for removing paints and cleaning (usually) metal surfaces.

To support regulatory initiatives, there is an urgent need for standard testing procedures (Vandermeersch et al., 2015) for the identification and quantification of MP in influent and effluent, preferably at an international level.

At EU level, the Marine Strategy Framework Directive (MSFD) is the dedicated binding legal instrument for assessing, monitoring, setting targets and reaching good environmental status (GES) with regard to marine litter (EC, 2008). A group including technical experts appointed by the Member States to support them in reaching GES for marine litter, co-chaired by JRC, has developed Guidance on Monitoring of Marine Litter in the European Seas and more recently thematic reports on sources of litter and on riverine litter monitoring (EC, 2013).

Several further notable regulatory initiatives have been implemented or are ongoing. These include:

- With regard to cosmetic products, some of which may contain microplastic/microbeads, the Regulation EC No. 1223/2009 (EC, 2009) stipulates that “a cosmetic product made available on the market shall be safe for human health”. The product should undergo a safety assessment, which takes into account the anticipated systemic exposure to individual ingredients in a final formulation.
- On 28 December 2015, the U.S. passed the ‘Microbead-Free Waters act of 2015’ to ban rinse-off cosmetics that contain intentionally-added plastic microbeads (from January 1, 2018), and to ban manufacturing of those cosmetics from July 1, 2017 (USC, 2015).
- The International Council for Exploration of the Sea (ICES), on request from the Oslo and Paris Commission (OSPAR), have developed common monitoring protocols from plastic particles in fish stomachs and selected shellfish (ICES, 2015).
- In 2015, the Danish Environmental Protection Agency published a report on the occurrence, effect and sources of release to the environment of MP in Denmark (Lassen et al., 2015).
- Initiatives and assessment by international organisation relevant to marine litter include the United Nations Environmental Programme (UNEP) which in 2014 adopted a resolution on marine plastic debris, noting the impact of marine litter (including plastics) on various areas, including potential risk to human health (GESAMP, 2015; UNEP, 2016).
- Canada: In June 2017, the Microbeads in Toiletries Regulations were published in the Canada Gazette, Part II: Vol. 151, NO. 12 – June 14, 2017 (Government of Canada, 2017). The regulation will prohibit the manufacture, import, and sale of toiletries used to exfoliate or cleanse that contain plastic microbeads, including non-prescription drugs and natural health products. For the purpose of the regulations, plastic microbeads include any plastic particle equal to or less than 5 mm in size. Plastic microbeads that are <5mm in size have been added to the list of toxic substances in Schedule 1 of the Canadian Environmental Protection Act (Government of Canada, 1999). A substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions that:
    1. Have or may have an immediate or long-term harmful effect on the environment or its biological diversity;
    2. Constitute or may constitute a danger to the environment on which life depends; or
    3. Constitute or may constitute a danger in Canada to human life or health.

1.1.3 Poorly soluble liquid droplets

Unlike NMs and MP that are solid particles, efforts to incorporate liquid substances that are poorly soluble into aqueous media (water solubility <1 mg/L at 20°C (ECHA, 2012)) may result in a dispersion of that liquid substance, forming liquid droplets. Further complications arise if the poorly soluble liquid is a substance of Unknown or Variable Composition, Complex Reaction Products and Biological Materials (UVCB), as is often the case with petroleum-derived products and essential oils. These UVCBs can have mixture components with varying levels of solubility associated with them (Letinski et al., 2016). As it is not always possible to measure the solubility of each component of a UVCB, guidance for test systems has been developed to measure and assess toxicity against a water accommodated fraction (WAF) (OECD, 2000; Singer et al., 2000; OECD, 2018). The WAF is the aqueous fraction containing the dissolved and/or suspended and/or emulsified fraction of a multi-component substance or mixture (OECD, 2000), with the explicit intention to avoid the formation of droplets of test substance. It is prepared in the lab using a low energy mixing process, which should be free of particles of the bulk material, while being representative of an environmentally relevant exposure scenario (Singer et al., 2000; Letinski et al., 2002). In these testing scenarios, droplets may act as a reservoir of test material and can physically interact with the test organisms.

In some cases, however, dispersions of poorly soluble liquids are intentionally tested. For example, when looking at the toxicity of crude oil spills that occur in the environment, physical processes, such as waves and currents, in addition to the use of dispersants used to mitigate the impacts of the oil spill, can all contribute to oil dispersions with widely varying droplet sizes. Various factors, such as the dispersion method, composition, etc., changes droplet size and longevity. The droplets act as reservoirs of the undissolved substance (Redman, 2015). Chapter 3 provides a more in-depth discussion on this topic. The presence of liquid droplets also explains measured aqueous concentrations of poorly soluble PAHs exceeding their theoretical solubility (Redman et al, 2012).

1.1.4 Harmonisation and learnings from engineered nanomaterial research

The observations of physical effects in a variety of standardised and non-standardised ecotoxicological aquatic test systems in relation to exposure to NMs and MP challenges our interpretation of the potential risks associated with the use of such materials. There are many stakeholders that have an interest in the results produced from toxicity tests. This results in a highly dynamic scenario that has the potential to limit the positioning and development of scientific test systems aimed at quantifying the differences between the intrinsic chemical toxicity, mediated by molecular interaction between dissolved substances and biological receptors, and physical effects associated with what is often mistermed 'particle-toxicity'. The example of MP is characteristic of a fast-moving issue that is likely to be regulated using hazard-based or precautionary approaches.

The adoption of hazard-based regulatory instruments, however, should act as a warning, in that they impede the development of scientific tools aimed at assessing toxicity and risk for a wide range of both naturally and synthetically derived particulate materials. In this report, we emphasise the importance of applying mechanistic understanding of the processes that influence exposure and effects of particles, differentiating between intrinsic chemical toxicity and physical effects. A key component in the development of mechanistic understanding is an appreciation of accurately defining the material under investigation, including the physical characteristics of size, shape, density, surface charge, as well as an understanding of its chemical nature (Chapter 2). Based on an appreciation of the complex relationship between intrinsic and extrinsic properties that can occur within a test system, standardised approaches for creating and maintaining dispersions of particles and poorly soluble materials (Chapter 3), and which are also compatible with the biology of the test organism (Chapter 4), are urgently needed to advance environmental hazard and risk assessment science for these materials (Chapters 5 and 6).

2. PHYSICOCHEMICAL CHARACTERISTICS OF SPARINGLY SOLUBLE MATERIALS THAT CONTRIBUTE TO EXPOSURE, UPTAKE AND EFFECTS

2.1 Introduction

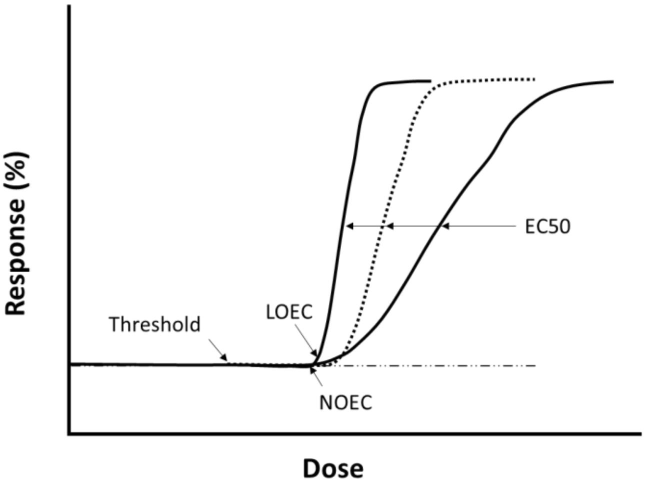
The environmental risk assessment (ERA) of chemicals involves consideration of a variety of physicochemical properties, theoretical principles, and characteristics of the test systems to be assessed. It is general practice to assess the intrinsic physicochemical properties of a substance in relation to adverse effects observed from standardised test systems.

The relationship between the concentration in the test system (i.e. exposure) and the observed effect can then be used to produce a dose-response curve. It is well understood that the toxicological response is the result of the chemical exceeding an internal threshold concentration (dose) that triggers a molecular initiating event, and which can cause adverse effects from the cellular to the organism level (National Academies of Sciences, 2017). Since quantification of the chemical at the site of toxicological action is typically not possible, the external medium concentration is often used as a surrogate, and effect responses are characterised by concentration-response relationships (McCarty and Mackay, 1993). In standardised aquatic toxicity tests, guidance requires that the test concentration does not exceed the solubility limit of the chemical, as observations are likely to relate to artefacts associated with undissolved test material, such as liquid droplets floating in or at the surface of the test solution or solid precipitate that might accompany the unrealistically high test concentration; and is unlikely to reflect the intrinsic toxicity of the test chemical (OECD, 2000; OECD, 2004; ECHA, 2011; U.S. Environmental Protection Agency (EPA), 2016).

If poorly soluble test chemicals (including MP, NMs, and poorly soluble liquids) are tested at concentrations exceeding solubility, physical interactions with the test organism may occur, and thus physical effects should be assessed and distinguished from intrinsic toxicity (if possible) to provide accurate input to the ERA. The potential for assessing physical effects associated with exposure to particulates are poorly addressed using traditional aquatic testing procedures. Additionally, the methods for controlling exposure of organisms to particulates are not well-prescribed in these procedures. Hence, the characterisation of the relevant adverse effect mode(s)-of-action for a specific chemical as well as accurately assessing the relevant and appropriate exposure in toxicity testing represents a major task for risk assessors and a fundamental element of chemical hazard and ERA in tests that exceed aqueous solubility.

The dose-response relationship is the link between the amount of a chemical taken up by a population and an observed adverse effect (OAE) in that population. The conventional dose-response curve (Figure 2) relates to the statistics associated with an OAE. In ecotoxicological aquatic test systems, a key objective of the study is to determine an endpoint metric based on the amount of chemical at which adverse effects are seen to derive a no observable (adverse) effect concentration (NOEC), a lowest observed (adverse) effect concentration (LOEC), and the effect concentration that has a 50% effect on the population (EC50), i.e. a 50% reduction in, for example, survival, growth, or reproduction. As illustrated in Figure 2, different dose-response curves can be obtained, depending on various factors, such as the endpoint being measured, the species, test conditions, etc., which can lead to variable results in toxicity data. Typically, the dose-response curve for the endpoint that results in a toxic response at the lowest concentration, i.e. the most sensitive endpoint, will represent the basis for deriving a predicted no effect concentration (PNEC), which is used in the ERA. There may be, for instance, several effects that might be perceived as being critical, dependent on the exposure scenario within the test system. The challenge associated with the evaluation of endpoints obtained from various studies for a specific chemical represents another major task for risk assessors. The primary goal is to determine the exposure level at which an intrinsic chemical toxicity is likely to occur.

Figure 2: Schematic illustration of conventional dose-response curve. The three different curves highlight the potential variability that might be observed, with the most sensitive response representing the result typically used in derivation of the predicted no-effect concentration (PNEC). Relative locations of the no observable (adverse) effect concentration (NOEC), the lowest observable (adverse) effect concentration (LOEC) and the effect concentration that has a 50% effect on the population (EC50) are also schematically represented.



Data generated for chemicals, based on standardised acute toxicity tests, for instance, have been used to directly link the intrinsic physicochemical properties of a chemical, such as its octanol-water partition coefficient (K_{OW}), to a lethal endpoint. A notable example is the relationship between K_{OW}, or the water solubility, of a chemical and baseline toxicity, which has resulted in the development of quantitative structure activity relationships (QSARs) (Mackay et al., 2014). Therefore, in instances where toxicological data may be missing for a chemical, estimates of its toxicity can be derived based on its toxicity classification, along with knowledge of its intrinsic physicochemical properties, and used within early screening and prioritisation stages.

The relationship between observed effects and the dissolved fraction of chemical thus represent an important basis for quantifying environmental risks. The assumption is that the dissolved fraction is homogenously distributed throughout the test system, ensuring that exposure of pelagic organisms occurs in a controlled, reproducible and optimised manner. It is therefore logical that the dissolved fraction is the primary focus of aquatic toxicity testing for model species (i.e. algae, *Daphnia*, fish).

In extrapolating the results obtained for sparingly soluble materials from standard test systems, however, several additional challenges begin to emerge. These challenges can be attributed to the applicability domain of standardised test systems, which have been designed primarily for chemicals where a freely dissolved amount can be taken up by the organism and become systemically bioavailable, leading to an intrinsic toxic effect. Thus, when testing poorly soluble chemicals and particulates, in excess of aqueous solubility, there is a need to differentiate between what might be an intrinsic toxic effect (E_{iT}) and an adverse effect associated with a physical interaction (E_{Pint}) with the substance in the test system (Donaldson and Poland, 2013; Skjolding et al., 2016). In this instance, the OAE may be the sum of intrinsic toxicity/toxicities and physical interaction(s) associated with the exposure scenario, i.e.

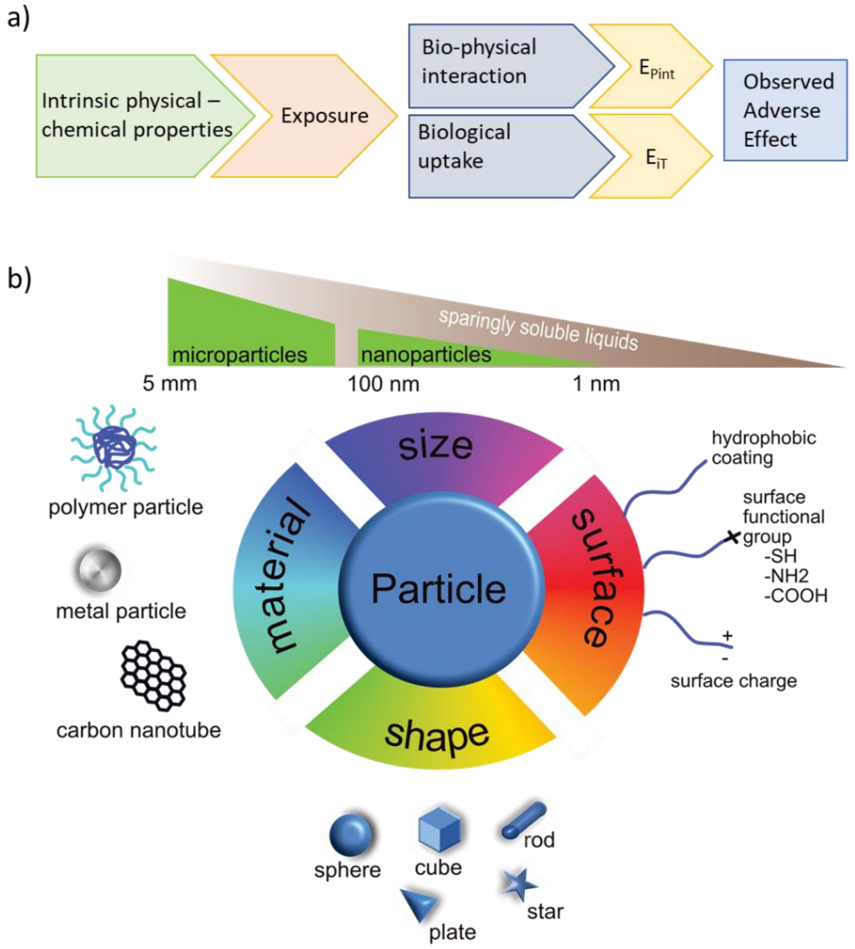
$$\mathrm{OAE} = \Sigma E_{iT} + \Sigma E_{Pint} \qquad \text{(Eq. 1)}$$

The above equation implies that there may be more than one E_{iT} and/or E_{Pint} that might contribute to the OAE. Mechanistic understanding that helps to better clarify and accurately define the ‘true’ toxicological mode-of-action that most strongly influences the OAE can only help to strengthen the ability to risk assess insoluble or sparingly soluble materials currently in use in commerce, as well as to help better inform future ‘benign-by-design’ innovation strategies.

It is notable that for measurably soluble chemicals the E_{Pint} term in Equation 1 is generally negligible, and that the OAE is entirely related to exposure to the freely dissolved fraction of that chemical, which results in an E_{iT} (see for instance (Skjolding et al., 2016; Hund-Rinke et al., 2018) in relation to NMs). Assessing the mechanistic toxicological mode-of-action of the chemical, however, is not a trivial exercise, it may differ between taxonomic groups and represents an important area of research that has resulted in the development of various toxicity classification systems (Kienzler et al., 2017) and the use of adverse outcome pathways (AOPs) as tools for providing additional insight (Ankley et al., 2010; Cronin et al., 2017).

In this chapter, we highlight several physicochemical characteristics that have been identified as being particularly significant in the development of standardised test systems for particulates, since they can strongly influence the accuracy and the OAE, and subsequently the interpretation of effect data in the chemical hazard assessment (Figure 3). Several important factors related to the effect testing of insoluble and sparingly soluble materials are important to consider, such as the particle size distribution of the particulates, their shape, crystalline state, hydrophobicity, rigidity, redox potential, specific surface area, surface charge, and chemical composition (Oomen et al., 2015; Sayre et al., 2017). Key questions to consider are: how do the physicochemical properties of the substance influence the ability to maintain exposure concentrations within the test system; and is it possible and necessary to differentiate and characterise OAE as being aligned to an E_{iT} and/or an E_{Pint}?

Figure 3: Illustrative representation of a) relationship between the intrinsic physicochemical properties and an OAE, and b) the physicochemical characteristics of particulates that have been identified as being important in the development of standardised systems for aqueous toxicity tests. Many of the properties represented for particulates will also be important with respect to liquid droplets of varying sizes that might form when testing sparingly soluble liquids, such as the size and shape of the liquid droplet. Characterisation of these properties will be important towards providing stronger interpretation of OAE.



2.2 Physicochemical characteristics

2.2.1 Particle size and shape

A fundamental structural characteristic of an insoluble material (particulate/liquid) is its dimension. The term 'particle size' is commonly used to compare solid particle or liquid droplet dimensions. Numerous techniques are available for measuring particle size and its distribution (Gao and Lowry, 2018). They differ (i) by the measurement principle which determines the metrics (number, volume, mass, intensity), (ii) by the detectable size interval, (iii) by the detectable concentration range and (iv) by the resolution of trace components in polydispersed suspensions (Gao and Lowry, 2018). Few techniques cover the entire size range relevant for sparingly soluble materials (laser diffraction perhaps representing the most universal technique), and very few provide chemical selectivity. It is difficult to examine size distribution of specific organic materials in a background containing other organics, often requiring some labelling or other distinguishing characteristic of the organic nanomaterial (Hu et al., 2016b; Laux et al., 2018).

Depending on the form or shape of the particle, the measurement of particle size and its distribution can differ, and results can vary dramatically between different sizing techniques (Gao and Lowry, 2018). Spherical particles can easily be quantitatively and unambiguously defined and measured through their diameter. However, many non-spherical particles cannot be fully characterised by a single dimension, even though most of the sizing techniques express the results in terms of an equivalent spherical diameter (Gao and Lowry, 2018). Such techniques give valuable information, but neglect shape information which could be crucial for interpreting uptake and effects in organisms. Alternative methods can be used that correctly evaluate the impact of particle shape on the size measurement, such as achieved by laser obscuration time (LOT) or image analysis, which gives access to a wide range of meaningful size and shape data (Gao and Lowry, 2018). Consequently, information about the sizing technique used is crucial for adequately comparing and interpreting ecotoxicological test results with non-spherical particles.

Quantification of the aspect ratio between particle lengths and diameters for needles, rods or fibres represents a more useful parameter in characterising the material tested, as opposed to defining an equivalent diameter. However, less information with respect to membrane permeability, ingestion or behaviour in the test solution can be derived from particle length alone (see for instance Chapters 3 and 4). In these instances, hydrodynamic transport is more strongly influenced by the diameter of the particle than on its length. Nonetheless, interactions of needles, rods or fibres with biological membranes may result in adverse effects that differ substantially in their modes-of-action as opposed to spherical particles (Choi et al., 2018). Consequently, it would thus be prudent to appropriately characterise the shape dimensions when attempting to better understand toxicological modes-of-action.

Depending on the chemical composition of the particle, the particle size itself can directly influence the dissolution rate of metal ions from NMs or the leaching of residual monomers or other chemical impurities associated with polymeric particles, as well as their dispersibility within the test system. Particle size can thus positively or negatively influence organism exposure levels. The influence of particle size on exposure can be further modulated by additional factors, such as particle-related properties (e.g., surface charge, surface area, density) and media-related properties (e.g., pH, ionic strength, natural organic matter (NOM)). For instance, He et al (2007) studied the particle size effect of 12, 32 and 65 nm hematite particles on particle aggregation. They found that at the same ionic strength, aggregation rates were higher for smaller particles. The hematite nanoparticles were identical in every aspect except for size; however, at pH 7 the smaller particles were less charged than larger particles, leading to lower electrostatic double layer repulsion forces and subsequently greater aggregation tendency (also see Section 2.2.2 - Surface charge, zeta potential). Similar results were obtained for 7, 10 and 20 nm citrate-stabilised silver nanoparticles in OECD test media used for short- and long-term toxicity tests with aquatic invertebrates (Römer et al., 2011). All particles aggregated quickly in the media with high ionic strength indicating the importance of media characteristics for dispersion stability. After diluting the media by a factor of 2, 5 or 10, aggregation was reduced, although the smallest particles (7 nm) were unstable under all media conditions (Römer et al., 2011).

For poorly soluble liquids, droplet size is related to the method of formation, i.e., via chemical or physical dispersion (Redman et al., 2017). The size of the droplet impacts its bioavailability, which is the argument for the use of dispersants to reduce the size and increase the bioavailability of droplets to encourage biodegradation during oil spills. Observed increases of oil toxicity following dispersion have been attributed to the size of droplets in dispersion (Bobra et al., 1989) and to increased dissolved PAH concentrations (which are known to be toxic to aquatic organisms) (Anderson et al., 1974b; Carls and Meador, 2009; Engraff et al., 2011). The presence of small droplets is expected to significantly increase the oil surface-to-volume ratio, increasing the partition rate of hydrophobic PAH components into water within their solubility limits, resulting in greater bioavailability in dispersed oil than in WAFs. Oil droplets can more easily interact and be transported by sediments, indicating that particulate matter and surfaces in aquatic testing systems can change the exposure profile of the material (Gong et al., 2014; Sørensen et al., 2014).

Particle size effects on the stability of dispersions can also influence the uptake and OAE associated with particle exposure. First, particle size determines the probability of ingestion by test organisms. For example, ingestion of MP has been reported for a wide range of biota; however, ingestion has been shown to be dependent upon the size of the particles and the test organisms, since the particles are usually mistaken for food. Particles are thus more likely to be ingested at sizes that are consistent with the size of the food ingested by a predator or consumer. This might also be relevant for NMs. While bioavailability of dispersed NMs is likely to be more efficient, nano-aggregates may be more efficiently ingested by test organisms (e.g., see Rosenkranz et al (2009)). However, the relationship between nanomaterial size and exposure/ecotoxicity is not yet fully understood. For example, the recent research project Nanogravur, could not find interrelations between size/shape and ecotoxicity (Hund-Rinke et al., 2018). This might possibly be explained by the fact that for some NMs the toxicity is mainly caused by the soluble metal ions, leading to an E_{iT}, rather than by E_{Pint} due to an exposure to the particles themselves.

Secondly, particle size determines the probability of particles to enter tissues and cells. For example, nano-sized MP have been observed to translocate from the gut into the tissue of mussels, crabs, and fish (Browne et al., 2008; von Moos et al., 2012; Watts et al., 2014; Pitt et al., 2018). Translocation of larger MP (>10µm) is not reported in the literature. In a study investigating the cytotoxicity of silver NMs in human cell models, elemental analysis of silver in cells by ICP-MS showed that smaller (5 nm) silver nanoparticles enter cells more easily than larger ones (50 nm), which is assumed to be the cause of higher chronic toxicity due to deleterious effects on the cell morphologies and membrane integrity through oxidative stress (Liu et al., 2010; Lopes et al., 2014). However, Albanese and Chan (2011) found a 25% decrease in uptake of aggregated gold nanoparticles with HeLa and A549 cells in comparison to single and monodisperse nanoparticles and a 2-fold increase in MDA-MB 435 cell uptake for the largest synthesised aggregates. These contrasting results suggest that the metal, the cell type, and the mechanism of interactions may play a significant role, and that the behaviour of aggregates with cells might have to be evaluated on a case-by-case basis. Finally, oil droplets can be of a size that they are not easily noted during test media preparation but can foul fish gills or entrap invertebrates, such as *Daphnia*, following standard test protocols, confounding impacts from chemical toxicity (Totten et al., 2003).

In addition to particle size, the shape of the particles might contribute to uptake and E_{Pint} with the aquatic organisms. For example, Ogonowski et al (2016) tested the effect of uniformly sized spherical polyethylene plastic particles (SPP), irregular-shaped polyethylene plastic particles (ISP) and kaolin clay on *Daphnia magna* mobility and reproduction using standard OECD tests, TG202 and TG211 (OECD, 2004; 2012b). The authors found no significant effects on daphnids exposed to SPPs and kaolin clay. The observation is important because it demonstrates the added value of using a reference material to compare effects. In this instance, the implication is that there is no difference in OAEs between SPPs and kaolin clay. By contrast, ISPs did exert negative responses in reproduction and survival, albeit at very high particle concentrations and relative contribution to the suspended material (Ogonowski et al., 2016). In addition, ISPs formed aggregates in *Daphnia* guts. Thus, the authors conclude that it is possible that formation and passage of aggregates through the gut might have caused internal damage (Ogonowski et al., 2016). Particle shape may also be relevant for the effect of particles which can enter cells. Forest et al (2017), for example, showed a significant impact of cerium oxide nanoparticle morphology on their *in vitro* toxicity with rod-like nanoparticles, as well as a dose-dependent enhanced pro-inflammatory and cytotoxic response that differed from cubic and octahedral nanoparticles. However, other studies with NMs found conflicting results. For instance, Wang et al (2015) observed lower toxicity of rod-like cerium oxide nanoparticles, as compared to cubic and octahedron-shaped materials, arguing that the higher surface area of the rods helped to reduce toxicity.

Lastly, the recently published OECD TG318 (OECD, 2017b) and its scientific background (OECD, 2012) provide guidance on sample preparation and dosimetry aimed at establishing stable dispersions of NMs in environmentally relevant aquatic toxicity test media. According to the guidance, particles <100 nm in an aqueous medium will undergo rapid Brownian motion with relatively small settling velocities. Brownian motion thus provides a mechanistic basis that can lead to a stable dispersion by counteracting gravitational settling forces, so long as the particles do not agglomerate (OECD, 2017b; Gao and Lowry, 2018). Particle density is also an important factor, for instance, it has been observed that particles of the same density will settle at different rates relative to their size, with larger particles settling faster than smaller ones (OECD, 2017b). When particles agglomerate, however, the effective density of the agglomerate becomes a controlling influence over sedimentation rates (Gao and Lowry, 2018). Particle size is thus one of a variety of factors that can influence dispersion stabilities, and therefore exposure. In the sections that follow, other factors, particularly the characteristics of the surface, will also be summarised. A key observation is that the various intrinsic physicochemical properties have the potential to interact with the test media in complex ways, which has resulted in the recommendation of a decision-tree matrix that aims at investigating the influence of various media-specific properties, such as ionic strength, pH, and concentration of organic matter on the dispersion stability of a specific NM (OECD, 2017b). Strengthened understanding of the relationship between intrinsic and extrinsic properties could thus be used to help prioritise and assess the quality of existing studies that are best fit for use within a regulatory context.

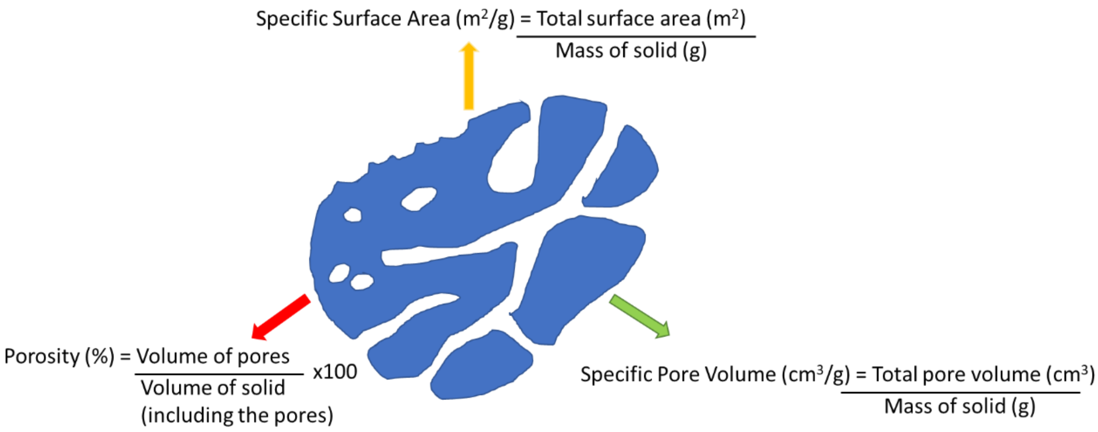
2.2.2 Particle surface

The particle surface provides the reactive interface between the particle and its local environment. Consequently, differences in surface properties can dramatically alter the environmental behaviour as well as the effect of particles on aquatic organisms (OECD, 2012). Surface properties, such as the specific surface area, the surface chemistry of the particle, and surface charge are important to characterise and quantify (Gao and Lowry, 2018). In this section, we summarise the importance of surface properties that influence both exposure and toxicity.

Specific surface area

Almost all solid materials have some amount of porosity associated with their physical state. Porosity is directly correlated with the specific surface area (SSA) of a particle, with non-porous particles having a low specific area that increases with increasing pore volume. Pores are classified according to their accessibility to an external fluid, with the possibility of both 'closed pores' and 'open pores' being present in a particle (Klobes et al., 2006). Closed pores are open voids within the interior of the particle and are not readily accessible to an external fluid. Nonetheless, the presence of closed pores can strongly influence the density, elasticity, and mechanical strength of a particle, and can therefore influence dispersion stability and exposure. Open pores are accessible to a fluid, whereby a fluid can pass entirely through the pore from one side of the particle to the other or follow a 'blind' path. Figure 4, adapted from Klobes et al (2006), schematically illustrates some of the key parameters associated with the porous nature of a particle, and which influence its SSA.

Figure 4: Schematic cross-section of a porous solid (adapted from Klobes et al (2006))



Both pore size and SSA are not amenable to direct measurement but are rather derived based on interpretation of sorption isotherms (Klobes et al., 2006). In general, gas adsorption measurements are used to characterise both the porosity and SSA of a particle. Nitrogen is the recommended adsorptive, but it may be necessary to use a variety of molecules to obtain a reliable assessment of the micropore size distribution (Klobes et al., 2006). In general, SSA is typically measured using the Brunauer-Emmett-Teller (BET) gas adsorption analysis method (Klobes et al., 2006), ECSA (electrochemical active surface area) based on cyclic voltammetry measurements (e.g., Jung et al (2016)) or USAXS (Ultra-Small-Angle X-ray Scattering) measurements in suspension (Thünemann et al., 2011). As an additional alternative, the determination of a volumetric SSA (VSSA) is recommended as a characteristic that may reduce variability in other derivations of SSA based on differences in densities between NMs (OECD, 2012).

Finally, the SSA of NMs has been shown to influence the dissolution or aqueous 'solubility' of a particle. A quantitative relationship that estimates the solubility product (K_{sp}) for NMs has been proposed:

$$\mathrm{Log}\,(K_{sp,SSA}) = \log\,(K_{sp,SSA=0}) + (2/3)\gamma(SSA)/2.303RT \qquad \text{(Eq. 2)}$$

Where $K_{sp,SSA}$ is the solubility product of a material with a specific surface area, $K_{sp,SSA=0}$ is the solubility product of the bulk material, γ is the solid/water interfacial tension, R is the ideal gas constant, and T is the absolute temperature (OECD, 2012).

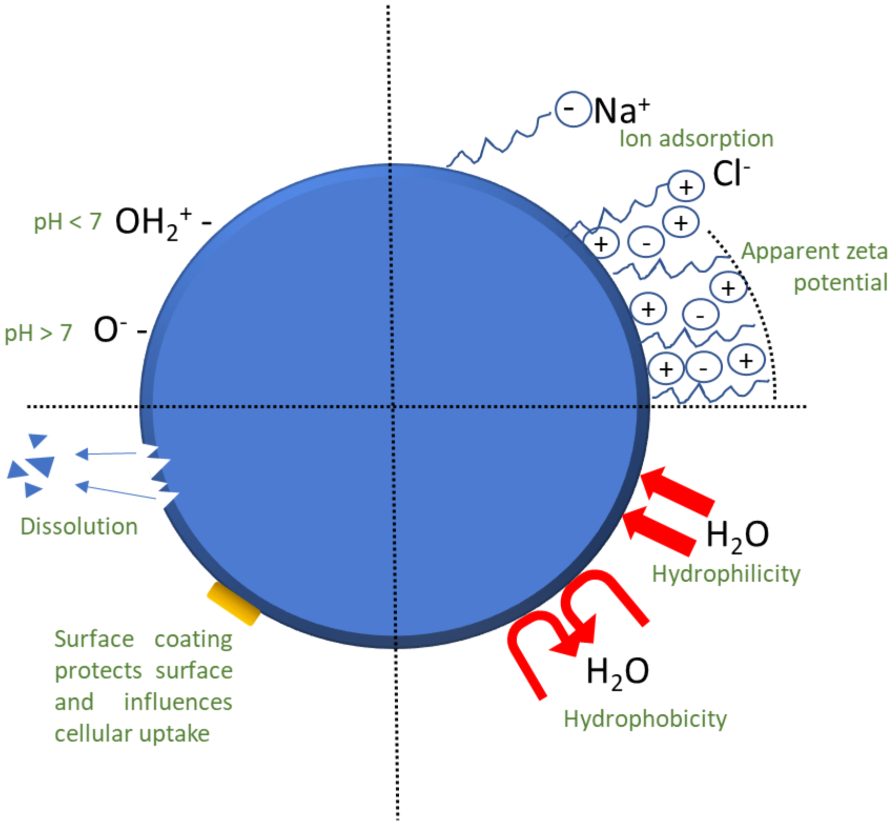
Characterisation of the SSA and porosity of a particle is important because of the influence these properties have on influencing sorption processes. Increasing SSA, for instance, increases the potential for particle aggregation and agglomeration, which can influence dispersion stability and bio-physical interactions due to increasing reactivity/sorption potential of the particles. Currently there exist several analytical challenges associated with derivation of SSA and porosity, which perhaps represents an important factor that has resulted in few studies directly correlating SSA and porosity to OAE. Nevertheless, there are a limited number of studies that directly relate particle SSA and porosity to OAE (see for instance Schmidt and Stöger (2016)).

Surface charge, zeta potential

It is worth noting that as particle size decreases the SSA of a particle increases, however, there is also an increase in the number of surface expressed molecules, which can lead to an increase in surface activity of the particle (Nel et al., 2006). The specific functionality of the surface groups strongly depends on the chemical composition of the particle, and can increase or decrease particle hydrophobicity, or cause the particle to be catalytically active or passive (Nel et al., 2006). In aqueous systems, reactive surface sites on the particle can interact with protons and other ions present in the system, or isomorphic substitution of an ion with one valence by an ion of a different valence in the crystalline structure can occur (OECD, 2012). Consequently, the surface charge of a given particle can be strongly influenced by the pH and ionic strength of the aqueous solution, which in turn will influence the dispersion stability and reactivity of the particles (Cosgrove, 2010).

As illustrated in Figure 5, particle surfaces are thus subject to various alterations in their intrinsic physicochemical properties relative to the properties of the extensive properties of the system they are in. For instance, NMs dispersed in an aqueous solution can result in surface ionisation and the adsorption of cations or anions that can generate a surface charge. This phenomenon is not strictly limited to NMs and can be observed for MP as well as for poorly soluble liquids, for which the critical micelle concentration can be altered (Cosgrove, 2010). Fotopoulou and Karapanagioti (2012), for example, studied the surface alteration of virgin and eroded polyethylene (PE) and polypropylene (PP) pellets in the marine environment, and observed a significant alteration of functional groups on the surface of PE. The formation of functional groups such as aldehydes, esters, carbonyls, ketones, and ketonocarbonyls, create an overall negative particle surface charge at seawater pH. In contrast, the particle surface charge of PP was observed to remain neutral (Fotopoulou and Karapanagioti, 2012). Particle surface modifications can thus vary substantially for different particle types.

Figure 5: Schematic illustration of intrinsic physicochemical properties in relation to extrinsic aqueous test system properties.



The particle surface charge can strongly influence dispersion stability. The surface charge creates a Stern layer over the particle in the dispersion medium, and a zeta potential is formed due to the phase differences of the medium and the surface of the particle (Cosgrove, 2010; Lowry et al., 2016). Zeta-potential is a physical property exhibited by any particle in suspension, and is the key criterion influencing dispersion stability, and is influenced by the ionic strength of the aqueous solution and the particle size (Harush-Frenkel et al., 2007; Zhang et al., 2009; Cosgrove, 2010; Lowry et al., 2016). Zeta potential is the electro-kinetic potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle and is generated from oriented solute molecules and ions surrounding the particle surface, as schematically illustrated in Figure 5. The stationary layer is composed of a layer of ions that are strongly bound to the particle (= Stern/Helmholtz double layer) and ions less firmly associated in a more diffuse layer, which screens the electric surface charge of the particle. Movement of the particle can result in shearing of ions from the less firmly associated layer (Lowry et al., 2016). The particle will exhibit an electric repulsion potential at the surface of the hydrodynamic slipping plane, which is the zeta-potential, measured in mV, and which cannot exceed 100mV.

As a measure of the degree of electrostatic repulsion between similarly charged particles in a test system, the magnitude of the zeta-potential is an important indicator of the stability of colloidal dispersions (Harush-Frenkel et al., 2007; Zhang et al., 2009; Cosgrove, 2010). A stabilised dispersion (kinetically stable when dispersed over a long time, but still thermodynamically unstable since colloidal dispersions always tend to aggregate and separate) describes a liquid where the particles may collide by Brownian motion or shear flow, but do not stick together after the collision by van der Waals attractive forces (Cosgrove, 2010). Particles with high positive or negative zeta-potential will resist aggregation and are thus electrically stabilised in the test solution (Lowry et al., 2016). Particles with low zeta-potential may adhere to one another and form aggregates, which can settle under the influence of gravity. The relevance of the zeta-potential for particle stability in biological test systems decreases with increasing particle size, since inertial effects of size and density increase in importance to any attractive van der Waals effects (Cosgrove, 2010). Consequently, zeta-potential can provide a useful metric for dispersion stability, which is particularly relevant to nano-sized particles (Lowry et al., 2016). Accordingly, zeta-potential measurements are recommended by scientists and regulatory agencies in the characterisation of the physicochemical properties of NMs for scientific and regulatory purposes (OECD, 2012; Sellers et al., 2015; Lowry et al., 2016).

The stability of the particles in a test solution in turn influences the pattern of exposure and uptake of the particles to test organisms. For instance, particles with low zeta-potential can form secondary particles (aggregates) of much larger size compared to the single particles, which can hamper oral/dermal uptake and thus reduce bioavailability to test organisms (Lowry et al., 2016). Bergami et al (2016), for example, evaluated the uptake and distribution of 40nm anionic carboxylated (PS-COOH) and 50nm cationic amino modified (PS-NH_2) polystyrene particles on brine shrimp *A. franciscana* using the larvae mortality test. Zeta potential measurements showed low values and confirmed their anionic (-9 mV) and cationic (+18 mV) surface charges. Dynamic light scattering (DLS) was used to measure the zeta potential of particles whereby the formation of large PS-COOH aggregates was observed, with PS-NH_2 resulting in less aggregation. The authors suggest that the high ionic strength of the medium screened the surface charge and interaction with naturally occurring colloids (e.g. NOM), which led to the formation of heteroaggregates for the PS-NH_2 particles. In contrast, stability was observed with strong negative charges on acrylic polymer particles in another shrimp study (Galloway et al., 2017). The importance of particle surface charge underlines the need to properly characterise stability in test media.

In addition, formation of aggregates can also reduce exposure of aquatic organisms by attachment of the particles to the walls of experimental equipment, the test organisms themselves or lead to enhanced sedimentation; however, depending on the test system, exposure may also be increased, e.g. in toxicity tests with sediment organisms where settled aggregates might be more bioavailable for the organisms tested. Thus, zeta-potential measurements can provide valuable information about the environmental fate, behaviour and toxicity of particles in the environment, although some caution is warranted given the complex interactions that can occur between the intrinsic physicochemical properties of the particle and highly variable extrinsic properties that occur in the natural environment (Lowry et al., 2016).

The zeta-potential is a calculated value based on measurements of electrophoretic mobility, and depends on various factors related to the particle, such as size, charge density or surface coating (e.g., polymeric functional groups, macromolecular protein corona, organic matter), as well as the dispersion medium, such as ionic strength, pH or presence of organic matter (Tielemans et al., 2006; Zhang et al., 2009; Cosgrove, 2010; El Badawy et al., 2011; Lowry et al., 2016). For instance, increasing ionic strength by adding salts to the test solution will lead to a more compressed stationary layer, which results in a lower hydrodynamic diameter. The particles can approach each other more closely and can be more strongly affected by attractive forces, leading to attachment of particles after collision. Highly negatively charged humic acids can differently affect the zeta-potential. Depending on the particle charge and the concentration of the humic acids the particle surface charge can either be reduced, neutralised or even provide them with a negative surface charge, which might stabilise the stability of the dispersion. For instance, Zhang et al (2009) observed that in the presence of natural organic matter (NOM) (2 mg/L) ZnO and NiO had negative zeta-potentials (< -30 mV), whereas the value was positive in the absence of NOM. The observations suggest that NOM can cause a negative charge on the surface of particles and increase their surface potentials (Zhang et al., 2009). In contrast, the zeta-potential of SiO_2 particles remained at a relatively constant value of -22 mV with NOM concentrations up to 10 mg/L (Zhang et al., 2009). Thus, NOM was useful in effectively stabilising ZnO and NiO particles in aqueous solution, while the high dispersion stability of SiO_2 was unaffected (Zhang et al., 2009).

The pH of the aqueous solution can also influence the zeta-potential since many particles show certain charge variability with the pH of the medium. Berg et al (2009) titrated TiO_2, Fe_2O_3, AL_2O_3, ZnO and Ce_2O from pH 12 – 2. Agglomerates were largest at the isoelectric point, which were near neutral, except for TiO_2 (IEP = 5.19) and Fe_2O_3 (IEP = 4.24) (Berg et al., 2009). Zeta-potential and agglomeration size increased over time for TiO_2 and Fe_2O_3, while Al_2O_3 and ZnO showed little change (Berg et al., 2009). Ce_2O increased in zeta-potential; however, the charge remained negative. The smallest agglomeration size throughout each particle suspension was found at the pH where the particle displayed a strongly charged surface (Berg et al., 2009). The observations suggest that the intense charge of the particles increases the repulsion between the particles, thus maintaining a more stable and monodisperse suspension (Berg et al., 2009).

As a general observation, positively charged particles tend to show stronger adverse effects in aquatic organisms (El Badawy et al., 2011; Della Torre et al., 2014) due to their electrostatic interaction with negatively charged cell surfaces (Lee et al., 2016) and ability to subsequently cause physical damage at membranes (Fabrega et al., 2009) or chemically induce reactive oxygen species (Choi et al., 2008; Ivask et al., 2014). However, evidence of particle assimilation into whole organisms is conflicting, as some studies report cationic particles easily entering cells (Harush-Frenkel et al., 2007), but these observations have not been confirmed in subsequent well controlled studies with whole organisms (Wray and Klaine, 2015).

Hydrophobicity

Hydrophobic chemicals, typically characterised by non-polar organic hydrocarbons, are commonly understood as chemicals that 'dislike' water. For organic chemicals that can be dissolved in water, their hydrophobicity can be understood as the balance between the free energy costs of cavity formation by the water molecules and the free energy gains due to the interaction of the organic chemical with the water molecules (Schwarzenbach et al., 2017). Consequently, the hydrophobicity of organic chemicals tends to increase with increasing molecular size, and will be maximal for chemicals that can only undergo van der Waals interactions with water (Israelachvili, 2011; Schwarzenbach et al., 2017). The hydrophobic forces that occur between particles and water are less well understood than for small molecules; however, it is generally understood that the force refers to a surface force rather than a force associated with the particle itself (Israelachvili, 2011). For instance, the attraction between hydrophobic particles in aqueous solutions is observed to be much greater than can be expected by van der Waals forces alone. Furthermore, water droplets have been measured to have large contact angles with hydrophobic particles (i.e. >90°), which can be used as a measure of a particle's degree of hydrophobicity (Israelachvili, 2011). The hydrophobicity of poorly soluble liquids is the reason that they form droplets in aqueous test systems. Increasing hydrophobicity correlates with a lack of bioavailability, which leads to the necessity of understanding the physical impacts of liquid droplets.

As a surface property of a particle, which influences its interactions with the surrounding aqueous environment, hydrophobicity thus represents another parameter that can influence dispersibility and exposure in aqueous test systems, and therefore influences OAEs (Gao and Lowry, 2018). The surface coating, either added intentionally, as for engineered NMs, or which might develop with time as a consequence of interactions with the constituents of an aqueous environment, is thus important to characterise when attempting to measure particle hydrophobicity (Xiao and Wiesner, 2012; Louie et al., 2016). Quantification of hydrophobicity can be achieved by measuring surface tension, the surface adsorption of a hydrophobic or hydrophilic molecular probe, or by an assessment of K_{OW} (Xiao and Wiesner, 2012). Advantages and limitations of the various techniques have been reviewed and summarised by Xiao and Wiesner (2012), who conclude that the adsorption probe method offers advantages over the other methods. Nevertheless, caution is warranted, as the most applicable approach may vary depending on the size range and chemical composition of the particles being investigated.

The fate and exposure of uncoated particles in aqueous systems can be understood based on characterising van der Waals and electrostatic forces incorporated in Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Louie et al., 2016). The adsorption of macromolecules onto the surface of particles requires modification to DLVO forces, as these can impart additional forces of interactions between particles, and can include steric and electrosteric forces, Lewis acid-base interactions (i.e. hydrophilic forces), and hydrophobic interactions (Petosa et al., 2010; Louie et al., 2016). The chemisorption (i.e. adsorption via a chemical covalent bond) or physiosorption (adsorption via non-covalent bond) of macromolecules to particles will result in increasing/decreasing hydrophilic forces or increasing/decreasing hydrophobic forces, and influences sorption to surfaces and biological uptake (Petosa et al., 2010; Louie et al., 2016; Mitzel et al., 2016). As with the other processes illustrated in Figure 5, the extrinsic properties of the aqueous environment will strongly influence the hydrophobicity of particles. The exception being those particles that have been engineered to include a hydrophobic or hydrophilic coating, which would represent an intrinsic property of the particle that should be characterised and quantified to better enable experimental control of aqueous exposures and interpretation of OAEs. Nevertheless, it is notable that even for engineered coated particles, the physiosorption of macromolecules can still occur and may negatively or positively influence exposure scenarios (Louie et al., 2016).

2.2.3 Particle chemical composition

The particle chemical composition is important towards defining many of the intrinsic properties of the particle, such as its density, crystallinity and solubility, and will strongly influence dissolution and particle dispersion within an aqueous system. Chemical analysis of the surface composition and the presence and location within the particle of impurities have also been recommended as being important to characterise and quantify, particularly in the context of NMs (Arts et al., 2015; NRCWE, 2015). Characterising the chemical composition, however, is not limited to NMs, and is similarly relevant towards the assessment of MP, and of poorly soluble organic liquids, some of which may be UVCBs. Consequently, the more information that can be characterised and quantified regarding the nature of the material to be tested for its aquatic toxicity, the better control of exposure and interpretation of OAEs.

The chemical composition of substances that might be tested for regulatory purposes can vary substantially. For instance, NMs used in commerce can either be carbon-based or inorganic in nature, amorphous or crystalline, coated or not coated. Similarly, MP can consist of a wide range of polymers, which might be linear or branched, consist of co-polymers or cross-linked polymers, contain plasticisers or other chemical additives, whereas poorly soluble liquids (water solubility <1 mg/L at 20°C (ECHA, 2012)), such as petroleum substances, tend to be characterised by complex chemical mixtures. Table 1 summarises a list of materials, which are representative of the variety of substances that would require analytical characterisation of their chemical composition, above and beyond what might accompany the characterisation of an individual chemical.

Table 1: Summary of commonly encountered materials used in commerce and characterised as NMs, polymers/microplastic, UVCBs.

Material type	Chemical formula	General comment
Carbon-based NMs		(Petosa et al., 2010)
Multi-walled nano tubes		
Single-walled nano tubes		
Fullerenes		
Fullerols		
Inorganic NMs		
Aluminium Oxide	Al_2O_3	Abrasives, ceramics, etc. (Krewski et al., 2007)
Cerium Oxide	CeO_2	Catalyst
Gold	Au	Nanomedicine, optics
Quantum Dots		Nanomedicine
Silica	SiO_2	Filler
Titanium Dioxide	TiO_2	Pigment, UV filter (Newman et al., 2009)
Silver	Ag	Anti-microbial agent
Zinc Oxide	ZnO	UV filter (Newman et al., 2009)
Zerovalent Iron	Fe	
Zirconia	Zr based	
Insoluble solid polymers		(Crawford and Quinn, 2017)
Polyamide (Nylon 6)	$(C_6H_{11}NO)_n$	Thermoplastic; semi-crystalline
Polyamide (Nylon 12)	$(C_{12}H_{22}N_2O_2)_n$	Thermoplastic; semi-crystalline
Polyethylene terephthalate (PET)	$(C_{10}H_8O_4)_n$	Thermoplastic; semi-crystalline
Polyethylene (PE)	$(C_2H_4)_n$	Thermoplastic; semi-crystalline
Polypropylene (PP)	$(C_3H_6)_n$	Thermoplastic; semi-crystalline (isotactic, syndiotactic); Amorphous (atactic)
Polystyrene (PS)	$(C_8H_8)_n$	Thermoplastic; Amorphous
Polytetrafluoroethylene (PTFE)	$(C_2F_4)_n$	Thermoplastic (fluoropolymer); semi-crystalline
Polyurethane (PU)		
Polyvinyl chloride (PVC)	$(C_2H_3Cl)_n$	Thermoplastic; amorphous
Poly(methyl methacrylate) (PMA)	$C_5H_8O_2$	Thermoplastic; amorphous
Acrylonitrile butadiene styrene (ABS)	$(C_8H_8 \cdot C_4H_6 \cdot C_3H_3N)_n$	Thermoplastic; amorphous
Polychloroprene (neoprene)	$(C_4H_5Cl)_n$	Elastomer; Semi-crystalline
Polycarbonate (PC)	$(C_{16}H_{18}O_5)_n$	Thermoplastic; Amorphous
UVCBs that are Poorly soluble liquids		
Petroleum substances		
Resins		
Essential oils		

It is notable that the materials listed in Table 1 reflect those that might be encountered in commerce, however, there exists considerable data pertaining to the aquatic toxicity of suspended solids (SS) that might naturally occur in the environment (Alabaster and Lloyd, 1982). It is also worth noting that many of the challenges associated with controlling for exposure and interpreting modes of toxicological action with aquatic test systems are consistent between research aimed at assessing the environmental risks associated with naturally occurring SS and the representative list of materials summarised in Table 1. Consequently, consistent with the discussion presented in this section, there is a need to characterise the chemical composition of naturally occurring suspended solids, which range from inorganic to organic (Bilotta and Brazier, 2008). Thus, it is anticipated that learnings can be achieved by considering the similarities and differences in the approaches adopted to address the various types of insoluble materials that aquatic organisms might encounter.

In the instance of NMs, many of which have always been present in the environment (Petosa et al., 2010), it is important to characterise relationships that exists between the chemical composition and the solubility, dissolution, or dispersibility of the tested material. Factors that might influence the aqueous solubility of a NM, for instance, are the chemical composition of the particulate and the particle coating. For many of the inorganic NMs, the dissolution of the metal ion, such as might occur for Zn, can lead to toxic effects associated with a freely dissolved fraction of the metal ion, as opposed to the E_{Pint} associated with the particle itself (Klaine et al., 2008; Lopes et al., 2014). As discussed previously (Equation 2), the dissolution rate is correlated to the particle SSA, which along with the chemical composition will influence the overall longevity of the NM in suspension. Whereas solubility of a chemical generally refers to the mass of chemical that dissolves in a unit volume of a solvent to form a saturated solution under specified conditions of temperature and pressure (Schwarzenbach et al., 2017), particulates by their very nature are generally characterised as insoluble or sparingly soluble. Thus, in their guidance on sample preparation and dosimetry for NMs, the OECD refer to the need to ensure a stable colloidal dispersion is formed (OECD, 2012), which refers to the mass proportion of a given sample of a NM that is held in solution as a colloidal suspension as a function of time.

The solubility and dispersibility of nanoparticles is strongly dependent on their composition and coating (López-Moreno et al., 2018). Most nanoparticles are either produced with a coating or capping agent, or they quickly acquire one when released into environmental matrices and biological systems because of their interactions with NOM, for instance, which can lead to the formation of a biofilm coating. The intentionally applied coatings often serve to reduce aggregation, thus increasing the dispersibility and stabilising the size of the nanoparticles. The coating composition varies depending on the nanoparticle and its intended application. For example, a metal-based nanoparticle may need a glutathione coating for increased hydrophilicity in a biological application. Some common coatings for metal-based nanoparticles are: polymers (for example, polyvinylpyrrolidone, chitosan, polyethylene glycol), citrate, humic acid, etc. Carbon-based nanomaterials, like fullerenes and nanotubes, can have surface functionalisation or coating, to alter their physicochemical properties, with molecules like polyethylene glycol or carboxylic groups.

For solid polymeric materials that are insoluble in aqueous solution, properties such as solubility and dissolution become less relevant to quantify. Polymers are molecules with three or more identical low molar mass units (monomers) that are connected covalently. Monomers are polymerised into macromolecular chains. In addition to monomers, other substances are often needed for polymerisation to occur, for instance, initiators, catalysts, and, depending on the manufacturing process, solvents may also be used. Further additives may be used to modify the properties of the parent polymer, including stabilisers, plasticisers, flame retardants, pigments, and fillers (Sperling, 2006; Verschoor, 2015). There are many different types of polymers that exist, but these can be generally divided into two broad groups:

- Thermoplastics (which soften when heated and can be remoulded); and
- Thermosetting plastics (which are cross-linked, do not readily soften and cannot be remoulded).

Polymerisation reactions are rarely 100% complete, and thus unreacted monomers, and in some cases reaction by-products and oligomers, may be found in the polymer, which may impart an E_{iT}, thus potentially influencing the interpretation of OAE. The proportion of unreacted monomers can vary greatly depending on the type of polymer, polymerisation technique, and techniques for reducing the levels of these constituents (Verschoor, 2015).

Solid synthetic polymers can be divided into three main categories (Goddard and Gruber, 1999; Sperling, 2006):

1. Plastics: which include thermosetting resins, such as urea resins, polyesters, or epoxies, and thermoplastic resins, such as polyethylene, polystyrene, or poly(propylene).
2. Fibres: which include cellulosics, such as rayon and acetate, and noncellulosic polymers, such as polyester and nylon.
3. Rubbers, for example styrene-butadiene copolymer.

Combinations of linear molecules, of linear molecules with cyclic molecules, and of cyclic macromolecules themselves can lead to a large number of variations in macromolecular structures, such as flexible coils, branched polymers, comb polymers, rigid rods, macrocycle, and star polymers or dendrimers (Goddard and Gruber, 1999; Sperling, 2006). Apart from naturally occurring proteins, all polymers are mixtures of many molecular weights. They are polydisperse, in that any polymer sample will be composed of individual polymer chains of varying size. How the polymeric molecules combine to form the solid structure of the particle is important to characterise, as their arrangement can influence the hardness and degree of crystallisation. For instance, polymers can be bound to each other in a regular geometric lattice to form a crystalline solid or combine irregularly to form an amorphous solid. The presence of branching or co-polymers can alter the formation to form a semi-crystalline solid, which is common for most plastic materials (Table 1). Characterisation of the polymeric structure of the particle, its glass phase transition temperature (T_g) and melting point temperature (T_m) have been proposed as important properties to consider when defining the physical state of the particle (i.e. difference between solid and semi-solid) (Verschoor, 2015). Finally, analogous to NMs, the dispersibility of solid polymeric particles within solution will be important to characterise and can be influenced by both the properties of the surface of the particle as well as the extrinsic properties of the test system. This issue is further explored in Chapter 3.

2.3 Conclusion

A consistent theme is presented in this chapter that works towards characterising the physicochemical characteristics of materials tested, and which emphasises differences and similarities between NMs, MP, and poorly soluble liquids. Knowledge of the physicochemical properties (both intrinsic and extrinsic) of the material and the extrinsic properties of the test system (Table 2) are key to characterise in controlling exposures within test systems that ensure an accurate assessment of E_{iT} for the material being tested. In the chapters that follow, examples for how the different types of information pertaining to the physicochemical characteristics of particles and poorly soluble materials are presented, with respect to exposure (Chapter 3), biological uptake (Chapter 4), and finally toxicity (Chapter 5).

Table 2: Summary of key physicochemical and test system properties (both intrinsic and extrinsic) important to characterise in controlling exposure in aqueous test systems.

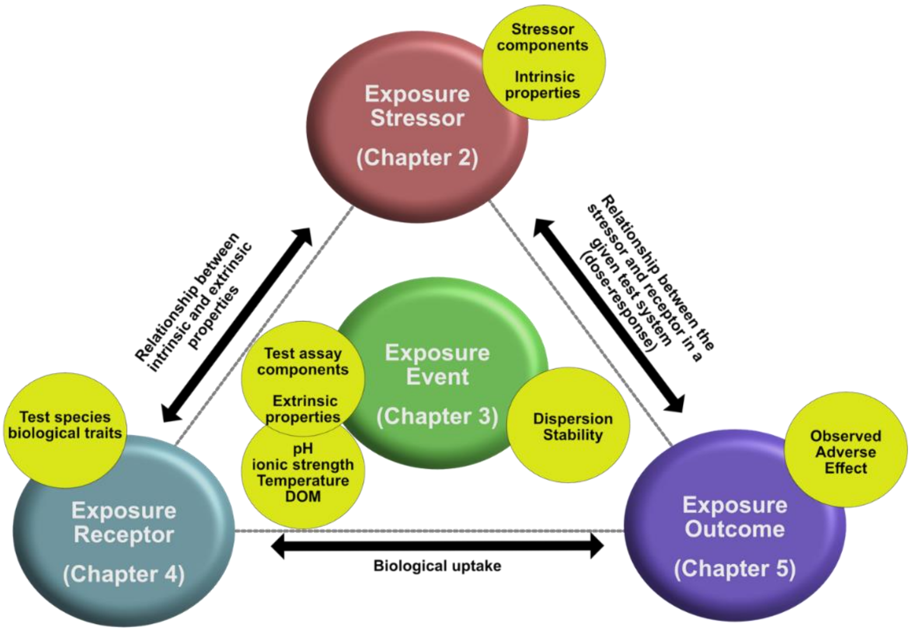
Intrinsic Properties	Extrinsic Properties
Solubility	(Petosa et al., 2010)
Density	Ionic strength
Specific Surface Area	Concentration of organic matter
Chemical composition/crystallinity	Volume of test system
Surface chemistry	Test duration/time

3. EXPOSURE

3.1 Introduction

Exposure, within the realm of ERA, describes the contact between a stressor and an organism in a test system (National Academies of Sciences, 2017). Exposure assessment focuses on characterising and quantifying a biological receptor interaction with an exposure to one or more environmental stressors of concern, resulting in E_{iT} and/or E_{Pint} to cause an OAE. For the purposes of ERA, exposure scenarios adopted in test systems should ideally consider ecological relevance in defining maximum exposure thresholds. When assessing the exposure, the magnitude, frequency and duration, along with characterisation of the receptor response are typically needed to estimate or quantify system-level responses to chemical perturbations (Mattingly et al., 2012). In their effort to strengthen the overall utility of exposure information that might be obtained from a toxicological study, Mattingly et al (2012) suggest an exposure science ontology that formally represents exposure concepts, the relationships between these concepts, and the relationship between exposure, susceptibility, and toxicological information. Figure 6 is a schematic illustration of the exposure ontology concepts and their relationships, which has been adopted from Mattingly et al (2012) to form the structure of the various chapters within this report.

Figure 6: Schematic representation of exposure ontology concepts (adapted from Mattingly et al (2012)) and their relationships with topics covered within the various chapters of this report.



The objective of this chapter is to summarise the relationship between the Exposure Stressor and the Exposure Event. The stressor, in the context of this chapter, refers to insoluble and partially soluble materials, consistent with those listed in Table 1, Chapter 2, whereas the exposure event refers to the properties of the test assay. A key message is that standard assay conditions are needed to systematically evaluate various factors that might influence the exposure outcome, i.e. the response. Consequently, this chapter will highlight the state-of-the-science with respect to methods used for controlling the exposure of materials within an aquatic toxicity test system, and where the interaction between intrinsic and extrinsic properties can influence the exposure scenario associated with the exposure event.

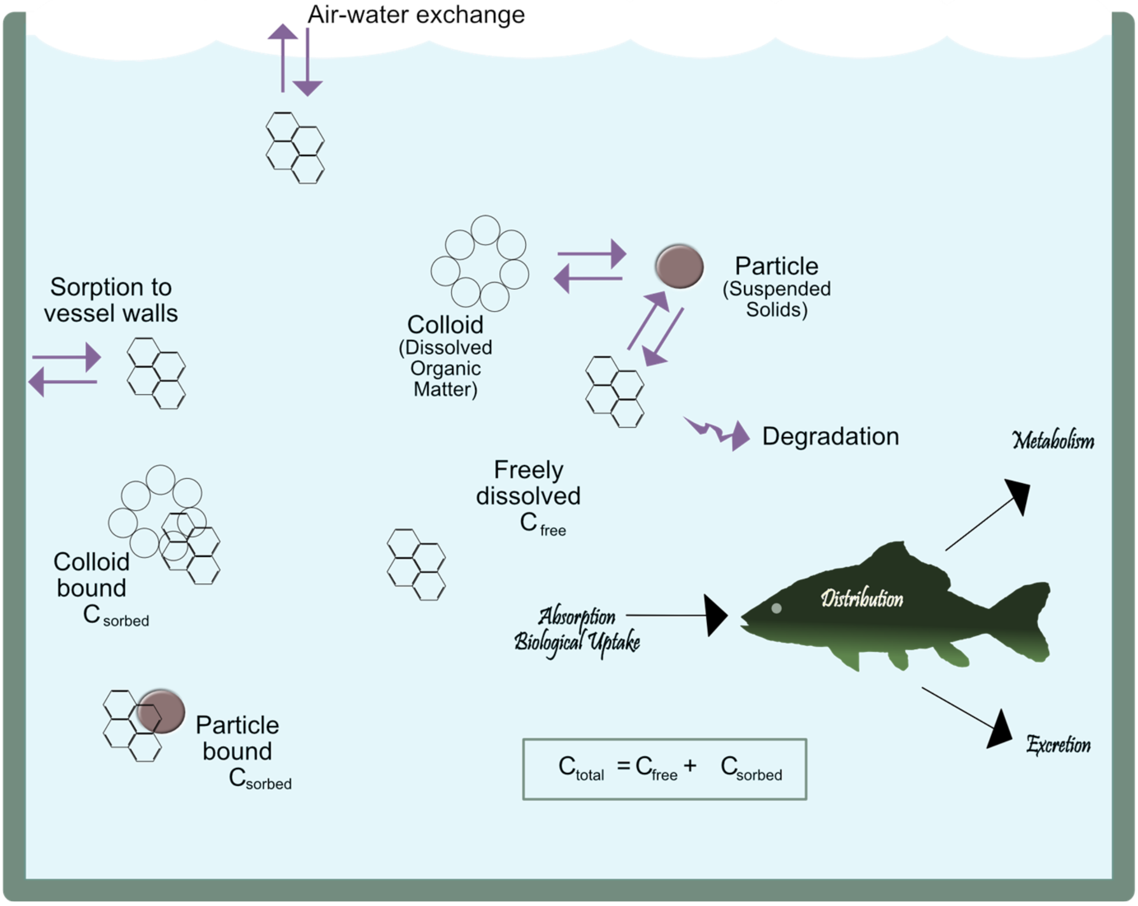
3.2 The exposure event

Aquatic toxicity testing of chemicals generally relies on the characterisation and quantification of the exposure event, as represented by the water concentration of the substance in the test system, and assumes that the OAE is caused by the chemical stressor accumulating in the tissues of the organism above a threshold level (Landrum et al., 2012). A widely accepted assumption is that the freely dissolved concentration of a stressor in the aqueous phase (C_{free}) (representative of the Exposure Event in Figure 6) is proportional to the internal organism concentration at the site of toxicological action (representative of the Exposure Receptor in Figure 6), which is also assumed to be proportional to the concentration in the tissues of the organism (C_{org}) (Escher and Hermens, 2002; Escher and Hermens, 2004; McCarty et al., 2011). In many instances, however, C_{free} may poorly reflect C_{org}, leading to the suggestion that significant improvements towards elucidating mechanisms of toxicity could be attained by directly measuring C_{org} (Kooijman, 1981; McCarty et al., 2011; Landrum et al., 2012; Mackay et al., 2014).

A key limitation of the assumptions described above is that C_{free} can be subject to time varying concentrations, causing the exposure event to change as a function of time (McCarty et al., 2011; Landrum et al., 2012). Changes in C_{free} can occur due to varying partitioning processes and reactions within the test system. For instance, it is well understood that hydrophobic organic chemicals will readily sorb to organic matter in the test system and/or vessel walls, whereas volatile chemicals will be subject to losses due to volatilisation from the test system (Kooijman, 1981). These partitioning processes can result in a significant decrease in C_{free}. Furthermore, toxicokinetic processes related to the absorption, distribution, metabolism, and excretion of the chemical within the organism can result in non-proportional differences between C_{free} and C_{org} (Kooijman, 1981; Landrum et al., 1992). Guidance to address these various challenges include direct measurement of C_{org} and/or the use of passive dosing or flow-through systems capable of better maintaining steady-state C_{free} of the stressor during the exposure event (Landrum et al., 1992; ECETOC, 1996; OECD, 2000; EC, 2003; ECETOC, 2003; OECD, 2004; Kramer et al., 2010; Smith et al., 2010; Engraff et al., 2011; McCarty et al., 2011; Adolfsson-Erici et al., 2012; Artigas et al., 2012; Landrum et al., 2012; Jager, 2014).

In their desire to strengthen the use of ecotoxicological data for ERA, Harris et al (2014) recommend the adoption of twelve principles of sound ecotoxicology. The fifth principle they present relates to defining the exposure, whereby it is emphasised that concentrations in the test system (i.e. C_{free}) be accurately measured (Harris et al., 2014). Unfortunately, their analysis of the peer-reviewed literature documents several examples where authors rely on reporting an assumed nominal concentration (C_{nom}), which has not been validated by chemical analysis, which thus results in increased uncertainty in the dose-response relationship presented in the study making ERA more difficult (Harris et al., 2014). Although it is noted that few studies quantify C_{free}, Figure 7, provides an illustrative representation of the various factors that might influence C_{free} in a test system. Given that the interpretation of an OAE relies on the assumption of a homogenous solution, whereby C_{free} is at steady-state, processes that significantly alter those conditions need to be adequately addressed and appropriately documented (OECD, 1992; ECETOC, 1996; OECD, 2000; ECETOC, 2003; OECD, 2004; Harris et al., 2014).

Figure 7: Illustrative representation of processes that influence the freely dissolved concentration (C_{free}) of a soluble chemical within an aqueous test system.



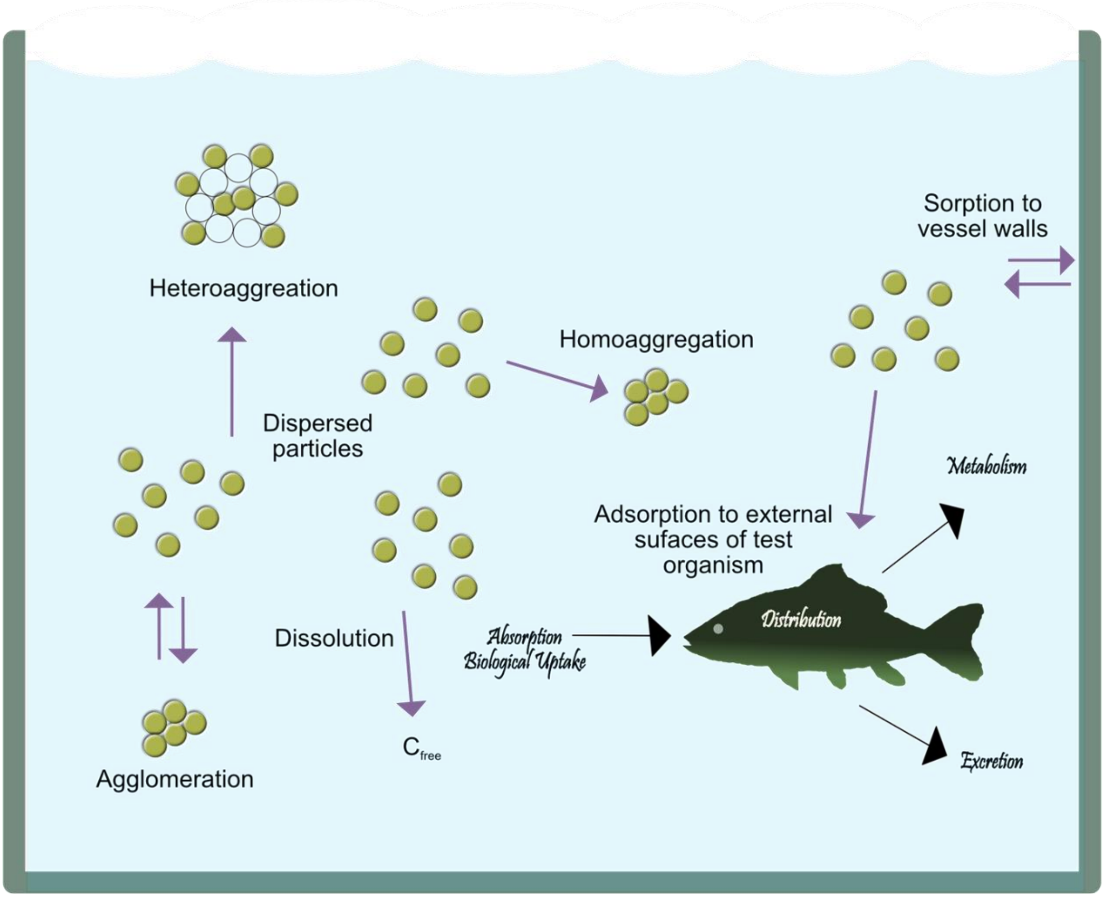
It is worth emphasising that the primary objective of chronic standard bioassays used for ecotoxicity is to provide information about the maximum concentration that gives *no effect* or an acceptable level of impact (e.g. NOEC, EC10, HC5) on a population relevant metric (such as survival, body growth, reproduction, population growth), which is consequently used to derive an environmentally 'safe' level (Kooijman et al., 1998). Characterising the exposure concentration from a bioassay (such as that depicted in Figure 7) is thus a critical element of the overall ERA. Although there are methods that have been developed to better maintain C_{free} within a bioassay, there are also methods available that are capable of interpreting data obtained from standard bioassays as a function of time. These methods use information about the rate at which OAE occur during the exposure event (Kooijman et al., 1998), and represent an important element of the discussions presented in Chapter 5.

While the challenges briefly summarised above for the testing of single chemicals in standard bioassays have similarities to those for insoluble and sparingly soluble stressors, there are some notable differences. The primary similarity relates to the fundamental principle of establishing a homogenous, steady-state exposure event, which requires the characterisation and quantification of the relationships between the intrinsic properties of the stressor and the extrinsic properties of the test system. For insoluble materials, such as some NMs and MP, as well as for sparingly soluble chemicals, quantification of C_{free} may not be possible and in exposures to dispersed material is not necessarily representative of a meaningful property with respect to bioavailability and biological uptake of particulates. A critical challenge, therefore, relates to defining a dose metric that best represents the exposure event. For insoluble materials this has proven problematic, in that the use of various exposure metrics has been communicated in the literature for both NMs and MP, with no standardised approaches currently available. For instance, for soluble chemicals C_{free} is reported with units of mass per unit volume, such as mol/L or µg/mL, but for insoluble materials the mass of particles in the test system per unit volume is often not meaningful, as the results for a material may not translate to another test of the same material of a different particle size. The relationship between particle size and the density of the material can thus result in significantly different exposure scenarios. As an illustrative example, Jeong et al (2016) report a relationship between accumulation and OAE on ingestion of MP in the monogonont rotifer *Barchionus koreanus* with respect to differences in MP size. The MP studied was non-functionalised polystyrene microbeads, with sizes of 0.05 µm, 0.5 µm, and 6 µm studied at mass per volume concentration ranging from 0.1 µg/mL to 20 µg/mL. A question not addressed in this study was whether differences in the number of particles in the test system influenced test results or whether results were 'actually' influenced by differences in the particle size, or a combination of the two. Based on the density of polystyrene it is possible to estimate differences in the number of particles between the different test systems, which implies that exposure to a 6 µm spherical particle at a concentration of 1 µg/mL results in approximately 8400 particles in the test system, whereas particle sizes of 0.5 µm and 0.05 µm result in about 1.5×10^7 and 1.5×10^{10} particles/L, respectively. Jeong et al (2016) report that OAE associated with particles of 6 µm were not significantly different from controls, but that OAE are reported for 0.5 µm and 0.05 µm at concentrations >1 µg/mL, or > 1.5×10^7 particles/L. Herein lies the difficulty in trying to utilise the results reported by Jeong et al (2016) in ERA, i.e. what is the relevant exposure metric and what is the environmental relevance of testing at >1.5×10^7 particles/L? The lack of consistency in the reporting of concentrations used in test systems, coupled with a lack of standardised approaches associated with an appropriate exposure metric to characterise the exposure event is a key issue identified by Connors et al (2017) in their appraisal of ecotoxicology studies of MP, and is an issue that is further addressed later in this chapter (Section 3.5) and in Chapter 5.

Further complicating an adequate assessment of the exposure in a bioassay, however, is the potential for insoluble and sparingly soluble materials to agglomerate and aggregate. In their recent guidance for dispersion stability of NMs, the OECD note that it is the number of particles that is most relevant in deriving the number of particle-particle collisions in a dispersion per unit time, and which determines the rate of agglomeration, not the mass of the particles in the test system (OECD, 2017b). Agglomeration refers to the process of contact and adhesion between particles dispersed in a solution that are held together by weak physical interactions and which leads to the formation of larger than colloidal size agglomerates that are capable of precipitating out of solution (OECD, 2017b). According to Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, the agglomeration of NMs depends on the particle diameter, Hamaker constant, ionic strength, and surface electrical potential, which is estimated from the zeta potential (ζ). The zeta potential of the particle varies with solution pH, and there is a pH at which the isoelectric point (IEP) is reached (i.e. ζ = 0mV) (Liu et al., 2011). Agglomeration is assumed to be a significant process near the IEP, as the electrical double layer diminishes and the attractive energy due to van der Waals forces more strongly influences particle-particle interactions (Jiang et al., 2008a; Liu et al., 2011). Particle agglomeration can be quantified using different computational methods or assessed experimentally (Jiang et al., 2008a; Liu et al., 2011; OECD, 2017b;

Abdolahpur Monikh et al., 2018). Indeed OECD provides guidance on an experimental method for qualitatively assessing the agglomeration of NMs (OECD, 2017b; Abdolahpur Monikh et al., 2018).

Figure 8: Illustrative representation of processes that influence the fate and behaviour of particulates in an aqueous test system.



Whereas particle agglomeration refers to a weak particle-particle interaction, which is reversible, particle aggregation refers to a stronger particle-particle interaction that leads to the formation of irreversible binding (OECD, 2017b). Two types of aggregation are possible: homo- and heteroaggregation. Homoaggregation refers to the binding of similar particles to one another, to form larger aggregates of the same particle, whereas heteroaggregation refers to the binding of dissimilar particles to form distinct aggregates. Figure 8 highlights the various particle-particle interactions that might occur within a test system. The importance of the extrinsic properties of the test medium (i.e. pH, ionic strength, characteristics and quantity of organic matter) and the particle number concentration itself are key components influencing the potential for particle agglomeration/aggregation (Petersen et al., 2015) and accordingly, the creation of a stable dispersion (Garner and Keller, 2014; Abdolahpur Monikh et al., 2018). In the following section (Section 3.3) the state-of-the art for creating stable dispersions is summarised for NMs, MP, and sparingly soluble liquid UVCBs.

Finally, Figure 8 illustrates the process of dissolution, which is a process associated with the degradation of an otherwise insoluble material. A key difference between exposures to particulates (Figure 8) and exposures in standard aquatic toxicity tests (Figure 7) is the dissolution process. The starting point and goal of standard aquatic tests is to obtain and maintain a soluble concentration of the test chemical. For clarity in this discussion, a fully solubilised substance is a substance in molecular or ionic form. Dissolution differs from solubility in that it also includes processes that result in the physical and possibly chemical degradation of the original test material. For exposures to insoluble materials, dissolution via degradation can represent a significant process, whereby characterisation and quantification of the dissolution kinetics (Equation 2, Section 2.2.2) is required to appropriately assess OAE associated with the exposure stressor (Rasmussen et al., 2018). The challenges associated with dissolution is also further explored in this chapter (Section 3.4) and in Chapter 5.

3.3 Methods for dispersion stability

In an aquatic toxicity test system, the potential exposure of the test organism to a stressor is promoted, often using procedures of questionable environmental realism. The main objective of the test system is thus to facilitate observation and quantification of OAE on the test organism. The standard practice when testing chemicals involves achieving a homogenous distribution of the dissolved test substance in the testing medium at concentrations that do not exceed the solubility limit of the chemical (Figure 7) and excluding any undissolved test chemical. For particulates tested as dispersions, this means maximising the number of encounters or the contact surface area between the test organism and the particulates. From a strictly mathematical perspective, optimisation of organism-particulate exposure is achieved by establishing a homogeneous dispersion of the particulates, keeping them in suspension using various methods discussed below. One caveat would be for dietary exposure, which is discussed in Chapter 4.

3.3.1 Dispersions of sparingly soluble liquids

The ERA of sparingly soluble liquids, such as those associated with petroleum products and other UVCBs, is primarily concerned with the bioavailable fraction of dissolved components of the complex mixture. It is well understood that the ERA of UVCBs is associated with several challenges, whereby characterisation of the individual components that comprise the complex mixture, many of which are often poorly soluble, represents an initial fundamental data gap to address (Dimitrov et al., 2015). As detailed in the OECD 23 guidance document for the aquatic toxicity testing of difficult substances and mixtures (OECD, 2000), several test methods are available that focus on the dissolved fraction. Given the complex mixture composition of UVCBs and the differing solubilities of the individual components, the utilisation of water-accommodated fractions (WAFs) or water-soluble fractions (WSFs) derived from a specific UVCB provides a viable option for testing the bioavailable fraction of these materials while minimising the potential for E_{Pint} from undissolved test substance. Establishing WAFs or WSFs involves stirring the test substance with the test media for a prescribed duration of time, sufficient to allow the insoluble fraction to come to equilibrium with the test media, followed by a settling period to allow phase separation of the insoluble fraction, then decanting or siphoning of the test media containing the solubilised test material (Singer et al., 2000). The suggested protocol for preparing WAFs further involves steps to minimise entrained droplets of test material, such as using an appropriate decanting method or reducing the speed of stirring to avoid the formation of droplets (Barron and Ka'aihue, 2003). An important point to consider when preparing WAFs for toxicity testing is that different components of the UVCB are likely to have varying water solubilities and consequently the composition of the WAF is not necessarily reflective of the whole test substance. Each constituent of the WAF will have its own partitioning process depending on the mole fraction in the mixture, the mass-to-volume ratio, and partition coefficients between the UVCB components and water. A minor hydrophilic constituent of an otherwise hydrophobic UVCB could represent the major test substance present in the WAF. As the chemical composition of the WAF is influenced by both intrinsic and extrinsic factors, WAFs must be prepared separately for each test concentration, not by dilution (ECETOC, 1996).[2]

There are several applications, however, in which the oil-water dispersion or emulsion may be the target of the ERA. One such example is in the assessment of dispersants for use during oil spills. In these cases, a chemically-enhanced WAF (CEWAF) is utilised to mimic dispersant use associated with an exposure event that might accompany an oil spill, with the CEWAF dispersion itself tested (Singer et al., 2000). However, caution in interpreting results based on the use of CEWAF is recommended, in that OAE associated with the dispersant or hydrocarbon can be obscured by the increased exposure to the hydrocarbons afforded by the dispersant, which may result in a false impression that the dispersant itself causes the OAE (Redman, 2015).

The ERA of petroleum substances that might be released following an oil spill accident can also be assessed by simulating physical processes that will result in their dispersion in the environment. Physically dispersed WAFs can be created using various methods. For the purposes of studies carried out after Deepwater Horizon (DWH), for instance, the creation of physically dispersed WAFs, including those for high-energy WAFs (HEWAFs), were standardised (Krasnec et al., 2016). HEWAFs were designed to physically create microscopic oil droplets for the purposes of assessing OAE associated with an exposure event consistent in the way the oil may be released (such as in DWH), or the action of currents, waves, winds, etc. on oil spills (Incardona et al., 2013). However, there is some debate as to the environmental realism of HEWAFs and their reproducibility (Sandoval et al., 2017).

The examples provided above are meant to be illustrative of the different types of exposure events that might be simulated within a laboratory environment and are not necessarily exhaustive of all scenarios in which emulsions or dispersions of immiscible liquids are deliberately created and tested.

Recently, an ambitious 3-year Cefic LRI project (ECO42: Fate-directed toxicity testing and risk assessment of UVCBs) has been initiated to develop and test new approaches for fate-directed ecotoxicity assessment of UVCBs based on a combination of analytical methods, dosing methods, fate-directed fractionation, and toxicity testing and models (Mayer et al., 2018). An important element of the project is to work towards a standardised method for dosing a UVCB into a test system. For instance, although the WAF method is recommended for controlling the exposure event in relation to testing UVCB substances, there exists some notable disadvantages, including challenges associated with maintaining steady-state water concentrations that result in a change in chemical composition as a function of time. Changes in the exposure event will occur due to differences in the rate that individual components of the UVCB mixture will bioaccumulate in the test organism, sorb to the walls of the test vessel and other organic material in the test system, and/or volatilise. Within the LRI ECO42 project, a method based on passive dosing will be developed as a potential alternative to using a WAF, and is proposed as representing a system that can better control steady-state conditions of an exposure event involving a UVCB mixture (Mayer et al., 2018).

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[2] The same logic applies for particles that may consist of multiple constituents or have minor soluble components, such as residual monomers, additives, plasticizers, which when in solution become bioavailable and may cause an OAE due to an EiT of the chemical at concentrations above a threshold level.

For other poorly soluble liquids, such as surfactants, the use of a WAF may also be applicable. However, aquatic toxicity testing of surfactants can also be carried out by constructing a dose-response curve with concentrations that do not exceed the critical micelle concentration (CMC), which can be perceived as being analogous to the aqueous solubility of an organic chemical. The CMC is defined as the concentration of a surfactant in water above which micelles spontaneously form and is dependent on the extrinsic properties of the test system, such as the ionic strength of the solution. Other intrinsic properties of the surfactant itself will also influence the CMC, such as the length of the hydrophobic tail and the hydrophilic head, whereby more hydrophobic surfactants tend to have lower CMC values than a surfactant that is more hydrophilic. Non-ionic surfactants, for instance, have lower CMC values than anionic and cationic surfactants because of differences in hydrophobicity.

3.3.2 Nanomaterial dispersions

Establishing and maintaining dispersions of NMs is a crucial and difficult step associated with their aquatic toxicity testing. NMs represent a wide variety of materials, varying in chemical composition and physicochemical properties. Consequently, methods to create and maintain dispersions need to be flexible to accommodate the different types of materials, while also providing sufficient standardisation to allow consistency in test results appropriate for regulatory acceptance (Hjorth et al., 2017). For instance, it is well understood that the use of different methods to prepare dispersions of the same NM can strongly influence OAE, with even small adjustments in test protocol resulting in large differences in the dispersion of the exposure event (Hartmann et al., 2015; Kaur et al., 2017; Abdolahpur Monikh et al., 2018).

Typically, a stock dispersion is prepared from a powder or liquid suspension. Caution is needed in establishing the dispersion, to avoid alteration of the intrinsic properties of the NMs, which might result in system-specific artefacts (Petersen et al., 2014). The complexity associated with developing a standardised method to accommodate such a wide variety of materials has resulted in the development and adoption of flowcharts aimed at providing standardised protocol (Hartmann et al., 2015; OECD, 2017b; Abdolahpur Monikh et al., 2018). Fundamental elements adopted in establishing a dispersion are summarised below:

- Characterisation of NM properties. Specifically, quantification of particle composition, chemical surface properties, water solubility, hydrophobicity, and particle morphology, such as the expected size of individual particles and agglomerates.
- Knowledge of the target concentration of the stock dispersion, which may be influenced by agglomeration and/or aggregation of the NMs and the testing medium.
- Characterisation of the properties of the dispersion in relation to the testing media, including volume, composition, and quality. It is important to ensure that the dispersion medium is compatible with the test material and organisms. For instance, a sterile suspension eliminates issues with confounding toxicity associated with bacterial contamination, whereas the ionic strength of the test medium can influence the exposure event, increasing or decreasing bioavailability of NMs, and consequently causing differences in OAE to occur, but low or high ionic strength media may be biologically incompatible with the test organism.
- The use of stabilising/dispersing agents require additional test controls to assess and differentiate OAE associated with the test NMs relative to the use of any additional chemical agent.

- NMs may require prewet powdered material to form a paste to overcome hydrophobic surface properties prior to dosing in the aqueous medium of the test system. As above, the inclusion of test controls may be required where solvents other than water are used.
- Application of an appropriate dispersion method, such as by ultrasonication or other mechanical process, should follow standard protocols.
- Adoption of appropriate quality control and quality assurance guidelines in monitoring the stability of the dispersion throughout the duration of the test, including documentation of all appropriate extrinsic properties of the test system, such as temperature, pH, ionic strength, etc.

It is worth noting that there have been several protocols proposed for standardising the dispersion process of NMs, which are based on information presented by Handy et al (2012), Hartman et al (2015), Coleman et al (2015), Kennedy et al (2017), Baun et al (2017), and the OECD (OECD, 2012; OECD, 2015a; OECD, 2017b), as well as proposed adjustments to OECD guidance (Hund-Rinke et al., 2016; Abdolahpur Monikh et al., 2018; Rasmussen et al., 2018). In most instances, the dispersion of NMs involves sonication, whether the origin of the particles is in a powder form or liquid suspension. Inter- and intra-lab reproducibility of sonication methods can be achieved by establishing consistency in the type of sonication applied, the probes used, the sonication settings, energy delivered, and sonication time (Taurozzi et al., 2012; Kaur et al., 2017; Kennedy et al., 2017).

Furthermore, most standard protocols focus on preparing a stock dispersion and subsequent dilution. However, if the NM is made of multiple constituents or has soluble impurities, then the methods employed in the preparation of WAFs for UVCBs are thus applicable. Specifically, the stock dispersion should be prepared separately for each concentration, not based on dilution, since it is well understood that OAE may be influenced by soluble impurities and/or constituents of the NM (Petersen et al., 2014).

3.3.3 Microplastic dispersions

The development and standardised application of test methods for establishing stable dispersions of MP in aqueous media are still under development. However, it has been widely acknowledged that based on the amount of research that has progressed towards the development of standardised dispersion methods aligned to NMs there are opportunities to expedite the adoption of appropriate methods for MP by incorporating knowledge that has been gained for NMs (Syberg et al., 2015; Hüffer et al., 2017; Rist et al., 2018; Rist and Hartmann, 2018). The current situation, which is characterised by a lack of availability and consensus of standardised methods related to dosing and maintaining stable dispersions of MP in test systems, represents a critical area of research to be addressed if data are to be consistently used within a regulatory context (Karami, 2017). Analogous to early testing of NMs, studies aimed at assessing OAE associated with exposure to MP have been typically characterised by poor reporting of key intrinsic and extrinsic properties of the exposure stressor and the exposure event, as well as inconsistent reproducibility within and between labs (Connors et al., 2017; Rist et al., 2018; Rist and Hartmann, 2018).

As discussed in previous sections, processes of aggregation and agglomeration can strongly influence exposure of MP within the test system. Consequently, characterisation of the physicochemical properties of MP, including both their intrinsic and extrinsic properties, is needed to better assess particle fate, which can be influenced by agglomeration, aggregation, and sedimentation processes. Establishing stable MP dispersions associated with an exposure event is critical for better interpreting the relationship between stressor, biological uptake, and OAE (Galloway et al., 2017; Karami, 2017). For instance, depending on the density of the MP tested, relative to its size and surface charge, it may be difficult to maintain stable dispersions of the particles. There appears to be a tendency for MP to either float or sink, which strongly influences the exposure event (Karami, 2017). For buoyant particles, the use of a low-density solvent, such as ethanol, to prepare stock solutions has been proposed as a method to help reduce the agglomeration of particles within the stock solution, and strengthen the accuracy of dosing (Karami, 2017).

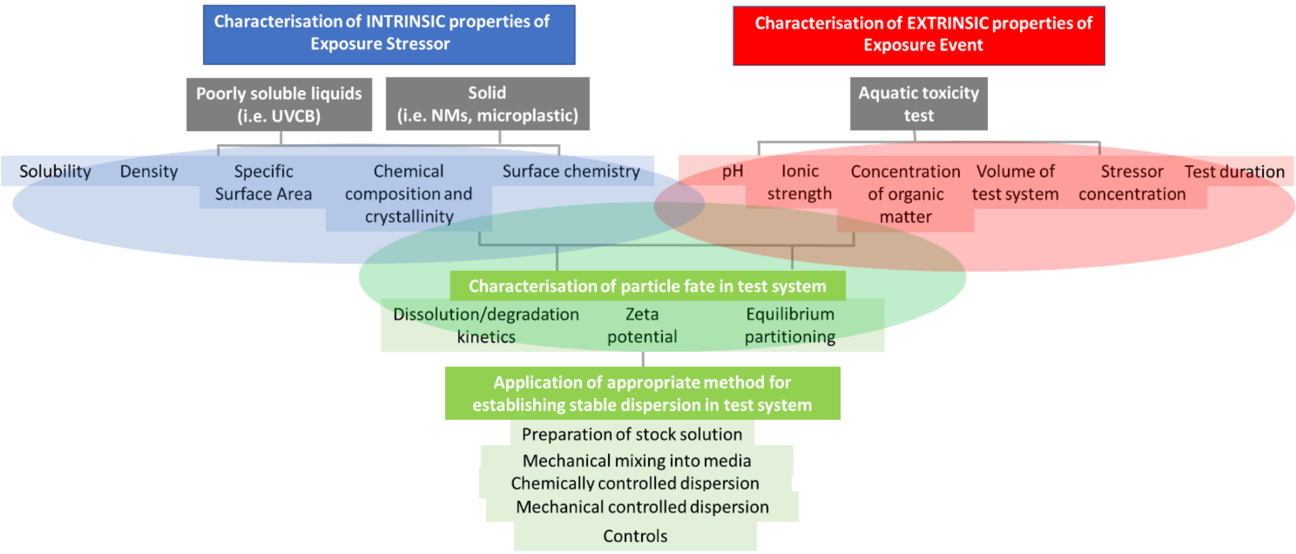
Establishing and maintaining stable dispersions in solution, however, may require the application of a pump to mechanically prevent particles from floating or sinking. Characterisation of the type and form of the polymer can be useful in helping determine the feasibility of the most relevant aquatic toxicity test, which would include the appropriate controls to characterise the influence of mechanical mixing and/or the use of solvents in maintaining dispersions (Karami, 2017). Consequently, and analogous to the testing of NMs, it appears reasonable that MP particle testing would greatly benefit from the development and consensus of standardised frameworks that enable consistent and transparent testing protocols.

3.3.4 Summary and discussion of particulate dispersion methods

An important observation in relation to establishing and maintaining stable dispersions of particulates in aqueous systems is an appreciation of the complex interactions between the intrinsic properties of the particles in relation to various extrinsic properties; of both the test system and the particles themselves (Abdolahpur Monikh et al., 2018). In their development of a decision-making framework for the grouping and testing of NMs (referred to as the DF4nanoGrouping), Arts et al (2015) utilise differences in intrinsic and extrinsic properties as a key element of their proposed groupings. The groupings they define are based around apical toxic effect endpoints, which note an obvious relationship between the hazard of a NM and its intrinsic properties (Arts et al., 2015). In this chapter we draw attention to the challenges associated with maintaining stable dispersions required for characterising the aquatic toxicity for both NMs and MP, which are strongly influenced by the complex interaction between the intrinsic and extrinsic properties of the particles and the test system. We also note the development and adoption of flowcharts aimed at establishing stable dispersions for NMs, which are largely based on a decision-making framework that attempts to consider the complex interactions that can occur between intrinsic and extrinsic properties. Figure 9 summarises the key elements that are typically included in such frameworks, which given the wide variety of materials and combinations of physicochemical properties and aquatic test systems available, can result in a wide array of complex frameworks. Consequently, it should be acknowledged that developing a standardised consensus framework for establishing stable dispersions in aquatic toxicity tests represents a non-trivial exercise, and one which would strongly benefit from input from all key stakeholders to:

- Agree on the environmental relevance of the exposure scenario to be used in the test system.
- Define the minimal set of properties to be characterised and reported in all aquatic toxicity test systems, of both the physicochemical properties of the exposure stressor and the exposure event.
- Agree on an appropriate exposure metric.

Figure 9: Summary of key elements of frameworks that have been proposed for development of best practices to establish stable dispersions of particulates and poorly soluble liquids in aqueous test systems.



This section thus highlights the key issues associated with creating and maintaining stable dispersions of poorly soluble liquids and particles. These dispersions need to be reproducible, representative, and stable enough to be manipulated. While it is not the objective of this report to address environmental realism, a certain amount of realism is needed for aquatic toxicity testing to ensure the results are appropriate and applicable for risk assessment purposes (Holden et al., 2016). Although there has been considerable resource directed towards establishing standardised protocols for creating nanoparticle dispersions, we note that there currently exists a lack of consensus regarding an appropriate method for particle dispersion within an aquatic toxicity test system. There are differing opinions, for instance, regarding whether stock solutions assist in the dosing of aquatic toxicity testing or whether they introduce too much uncertainty.

Once a dispersion is created, there are still several confounding factors to address, some of which can impact the stability of the dispersion. Some of the available methods for creating an aqueous dispersion can help with issues of stability and include the use of capping agents, solvents or certain additive dispersants (such as bovine serum albumin (BSA) and natural organic matter (NOM)) (Petersen et al., 2014; Cerrillo et al., 2017; Abdolahpur Monikh et al., 2018). Alternatively, dispersion stability can be achieved by adjusting the test system pH and/or ionic strength. In such instances, the stability of the dispersion is achieved by manipulating the electrostatic charge of the nanoparticles by controlling the pH to reduce agglomeration near the isoelectric points of the nanoparticles (Abdolahpur Monikh et al., 2018).

The use of mechanical methods to maintain particles in suspension, on the other hand, can sometimes lead to the opposite effect, as the increased movement of the particles can increase particle collision rates and agglomeration (Hüffer et al., 2017). Consequently, avoiding agglomeration requires a method that helps to reduce the collision frequency of the particles, which can be achieved by performing studies at lower particle concentrations. The stability of the dispersion in the presence of the test organism should also be considered (Filella et al., 2008). It is possible that the predominately negatively-charged surface of an organism can serve as a site for sorption of particles, particularly those with a cationic surface charge (Casado et al., 2013). Given the various methods that can be adopted to establish and maintain particle dispersions, it is highly recommended that interpretation of OAE follow fundamental principles of ecotoxicology through the inclusion of appropriate negative and positive controls (or control experiments), which can further aid in improving mechanistic insight with respect to toxicological mode-of-action associated with the OAE (Casado et al., 2013; Harris et al., 2014; Connors et al., 2017).

We anticipate that the elements of the framework presented in Figure 9 will thus likely represent important parameters in defining the minimal set of properties to be characterised and reported in all aquatic toxicity test systems, of both the physicochemical properties of the exposure stressor and the exposure event, a tool for assessing the hierarchy of impact for these individual characteristics, and how the properties influence the quality of data produced in ecotoxicology studies for risk assessment purposes. It should also be noted, however, that the challenges highlighted in this section with respect to creating and maintaining particle dispersions are also perhaps reflective of challenges with addressing different questions. Test design, therefore, should consider how the data obtained from a study might be used, and should therefore be fit-for-purpose. For instance, efforts to ensure dispersion stability within a test system are likely to benefit interpretation of data for strengthening mechanistic understanding of OAE. However, results may not accurately reflect environmentally relevant conditions, whereby the presence of stable dispersions will be rarely encountered. Consequently, careful consideration of how data are to be used will be required.

3.4 Dissolution and degradation

For poorly soluble liquids, the very act of creating droplets can increase the rate of dissolution of the constituents by increasing surface area. As mentioned in Chapter 2, PAH concentrations appear to increase with oil droplet formation (Anderson et al., 1974b; Carls and Meador, 2009; Engraff et al., 2011). For some solid particles, dissolution and degradation of the particle can be an important factor to consider. Dissolution involves the release of ions from a particle, even within an aggregate, and is a surface-controlled process that is dependent on the particle surface area and the concentration of the dissolved ions near the surface of the particle (Quik et al., 2011). Consequently, the resulting extent of dissolution is governed by the thermodynamics of the test system; i.e. if the overall free energy is negative, dissolution will occur where the solubility product coefficient (K_{sp}) is positive (Garner and Keller, 2014). If the released ions, such as Ag^+, can react readily with constituents of the aqueous solution, such as Cl^-, near the surface of the particle, a high thermodynamic gradient can result, which will lead to faster overall dissolution (Garner and Keller, 2014). Thus, similar to agglomeration/aggregation, the dissolution of a material depends on its physicochemical properties, such as chemical composition, particle size, shape, crystallinity, and surface modification, as well as the extrinsic properties of the test system, including pH, ionic strength, constituent solvated molecules, temperature, and concentration (Misra et al., 2012; Avramescu et al., 2017).

In their review of dissolution rates for various NMs, Garner and Keller (2014) summarise dissolution time frames as being generally on the order of weeks to months for several different types of NMs and aqueous systems, with an exception of ZnO, where dissolution can occur within hours in either freshwater or marine systems. Given the toxicity that is typically associated with the metal ion of NMs, characterisation and measurement of dissolution rates in the relevant solvent should therefore be quantified (Utembe et al., 2015).

Whereas dissolution can alter the physicochemical properties of particles being tested, other abiotic and biotic transformation processes can also lead to changes in both physical and chemical properties of the material. Significant degradation processes include oxidation, sulfidation, and reactions with phosphorous (Garner and Keller, 2014). Once again complex interactions between the intrinsic properties of the particle relative to the extrinsic properties of the test system will govern the overall importance of any potential degradation process and should therefore be taken into consideration throughout the design of the test and exposure system.

For MP it is well understood that a variety of degradation processes encountered in the environment result in the plastic particles continuously decreasing in size as a function of time (Barnes et al., 2009; GESAMP, 2015; Gewert et al., 2015). However, within the duration of most aquatic toxicity tests it is unlikely that significant transformation will occur.

Depending on the age of the plastic and the manufacturing process, the presence of chemical additives to the plastic polymer, such as plasticisers, and/or unreacted monomers, should be characterised and measured (Rist and Hartmann, 2018). Analogous to the dissolution of metal ions, which can cause E_{iT} effect at the molecular level, freely dissolved chemicals associated with MP can also elicit an OAE that is not necessarily due to the particle itself, but a sample artefact. It is thus prudent to fully characterise the sample material to address concerns of dissolution, degradation, and the presence of contaminants to strengthen interpretation of dose-response curves associated with an OAE (Rist and Hartmann, 2018).

3.5 Analytical quantification

When dosing soluble chemicals to an aquatic toxicity test, the exposure concentration should not exceed the aqueous solubility for the chemical (or saturation concentration in test medium). The solubility limit thus represents the maximum concentration and is thus a prerequisite parameter needed to establish test conditions in an aquatic toxicity test (OECD, 2000; OECD, 2004; ECHA, 2011). Aqueous solubility is defined as the saturation point of the mass concentration (units of mass per volume of solution) of a chemical in pure water at a given temperature, typically 20°C (OECD, 1995). Depending on the properties of the chemical (i.e. neutral organic, ionisable organic, inorganic, metal, etc.), a wide variety of chemical analytical techniques can be used to quantify the functional solubility of a chemical in a test system based on the use of standardised OECD test protocols, such as the OECD 105 (OECD, 1995).

For sparingly soluble UVCBs and particles dosed within an aquatic test system, application of a solubility limit for the substance does not represent a useful parameter in setting up the test system.

Mixtures of organic chemicals, such as petroleum substances, behave differently than their single constituent compounds when dosed into an aquatic system. Given the variable hydrophobicities and solubilities of components that might be associated with a UVCB substance, water solubility is thus not applicable. For these complex materials, the exposure event is established by enabling the testing of the composition of the aqueous solution formed at equilibrium under a defined set of conditions, such as using a WAF, CEWAF, or HEWAF, as described above (Section 3.3). Typically, the concentration of the soluble fraction of poorly soluble liquids is the metric of interest (Singer et al., 2000). The practicality of analytical verification of test concentrations in test media, however, can vary greatly, depending on the test material. Petroleum substances, for instance, are amongst the most challenging to analyse, given that they are UVCBs and can be very poorly soluble, often near the detection limits of available analytical instruments and may require alternate metrics (not just mg/L). For example, the solubility of C9-C18 alkanes was assessed using a slow-stir technique and the recommended metric was solvent accessible molar volume (Letinski et al., 2016). It may not always be possible, however, to establish that equilibrium of all mixture components has been achieved, given the associated analytical challenges with measuring low solubility chemicals; in these instances, the time and type of agitation of the test vessel must be thoroughly described to enable reproducibility of the test (ECHA, 2011). If the concentration of multiple individual constituents is determined then other practical challenges arise, such as determining if one or multiple constituents are responsible for the OAE, and thus how to express the effect concentration. Factors such as the loading or the nominal concentration of the UVCB into the test system, which is often a WAF, must be reported. This is usually done by weight per volume metrics (mg/L) or volume per volume (mL/L). In instances where liquid droplets are formed, characterisation of the physicochemical properties of the droplets is helpful to assess their variability and behaviour, which might either result in droplets that are persistent or ephemeral. One option is to extract the liquid droplets into an appropriate solvent for subsequent measurement (Incardona et al., 2013; EFEO/IFRA, 2016).

Quantifying dispersions of particulates in aqueous test systems can be assessed by several different methods, which will also give information on particle size and particle size distribution, and include the application of visualisation tools via microscopy, analysis of turbidity (i.e., light scattering or spectroscopy), or separating particles based on size using chromatography (e.g., field flow fractionation). A more thorough comparison of the various techniques is discussed in Rasmussen et al (2018).

Whereas there exists a variety of analytical methods to characterise and quantify particle dispersions within an aqueous test system, a key question arises on how to establish threshold toxicity values in metrics that can be consistently applied to different types of compounds, such as between soluble chemicals and insoluble particles (Hidalgo-Ruz et al., 2012; Claessens et al., 2013; Connors et al., 2017; Karami, 2017). Depending on the analytical method applied to characterise and quantify particle dispersions, exposure metrics can be reported using various metrics, such as mass/volume, particles/volume, particles/area, and for testing involving solid matrices, such as sediments or biological tissues, mass/mass or particles/mass (Hidalgo-Ruz et al., 2012). In any case, the exposure metric should have a mechanistic relationship to the OAE, and not be chosen simply based on available analytical methods. The variable units in which abundance and mass of particles are reported is problematic when comparing exposures between different studies, although in some instances units can be converted based on assumptions of density and other intrinsic and extrinsic properties (Hidalgo-Ruz et al., 2012; Claessens et al., 2013; Connors et al., 2017; Karami, 2017). Consequently, detailed information regarding the intrinsic and extrinsic properties of both the exposure stressor and exposure event (such as highlighted in Figure 9) are required to enable unit conversions. Alternatively, consensus and/or standardisation regarding the analysis and reporting of exposure metrics within aquatic toxicity tests would greatly strengthen interpretation of test results, and is suggested as a fundamental element towards strengthening mechanistic understanding of OAE (Hidalgo-Ruz et al., 2012; Connors et al., 2017; Rist and Hartmann, 2018).

3.6 Conclusion

When performing an aquatic toxicity test the development of standard methods for establishing, maintaining, and characterising exposure are fundamental elements towards strengthening mechanistic understanding of OAE. This chapter has highlighted the importance, as well as the challenges and limitations, of establishing and maintaining homogenous steady state exposures for sparingly soluble and particulate materials, which can be dependent on both intrinsic/extrinsic properties of the exposure stressor and extrinsic properties of the test system. An important observation relates to the similarities between MP and NMs, whereby considerable learnings can be extrapolated between the development of methods for each group of particles. It is further suggested that the development of methods aimed at controlling exposures within aquatic toxicity test systems for NMs and/or MP, consider implications to a broader range of particles, both natural and synthetically manufactured, in that it is likely that improved testing for particulates and sparingly soluble materials can be developed based on the learnings obtained thus far. To facilitate the learning process and ensure the development of standardised methods to be used within a regulatory context, it is recommended that constructive communication between key stakeholders be supported.

The following summarises several outstanding issues that would benefit from stakeholder engagement:

- Development of clear, common definitions for particle categorisation and exposure metrics.
- Consensus on reporting requirements aligned to both the exposure stressor and exposure event, including particle intrinsic and extrinsic properties and test system extrinsic properties most likely to influence aggregation, agglomeration, sedimentation, dissolution, etc.
- Inclusion of chemical leaching controls (monomers, chemical additives, etc.).
- Development of protocols for creating and maintaining dispersions, sample preparation, and analytical methods to minimise test artefacts and strengthen reproducibility and interpretability.
- Standardised methods to assess environmental transformation processes, such as assessing OAE associated with exposure to environmentally relevant aged MP or NMs.
- Development and use of standard reference materials for method validation and test control.
- Research aligned to identifying and prioritising environmentally relevant exposure scenarios and/or benchmarks.

4. BIOLOGICAL UPTAKE

4.1 Introduction

The biological uptake and accumulation of chemical substances is a dynamic process that can include several uptake, elimination, and depuration routes acting simultaneously. Concerns related to the bioaccumulation of hydrophobic organic chemicals are due to their passive diffusion across biological membranes and subsequent accumulation in lipid-rich tissues. This process has been shown to be thermodynamically controlled, and can be modelled based on knowledge of the equilibrium partitioning coefficients of a chemical between a biological tissue and water, and the tissue volume fraction, such as by:

$$K_{bw} = f_{lip} \cdot K_{lipw} + f_{prot} \cdot K_{protw} + f_{lig} \cdot K_{ligw} + f_{cut} \cdot K_{cutw} + \ldots \qquad \text{(Eq. 3)}$$

Where K_{bw} is the whole organism to water partition coefficient, K_{lipw} is the lipid to water partition coefficient, K_{protw} is the protein to water partition coefficient, K_{ligw} is the lignin to water partition coefficient, K_{cutw} is the cutin to water partition coefficient and f_{lip}, f_{prot}, f_{lig}, and f_{cut} are the volume fractions of lipid, protein, lignin and cutin in the organism, respectively (Schwarzenbach et al., 2017). Research that characterises and quantifies the various parameters of the above equation are helpful towards developing greater mechanistic understanding of key processes influencing the uptake and accumulation of organic chemicals in biological organisms.

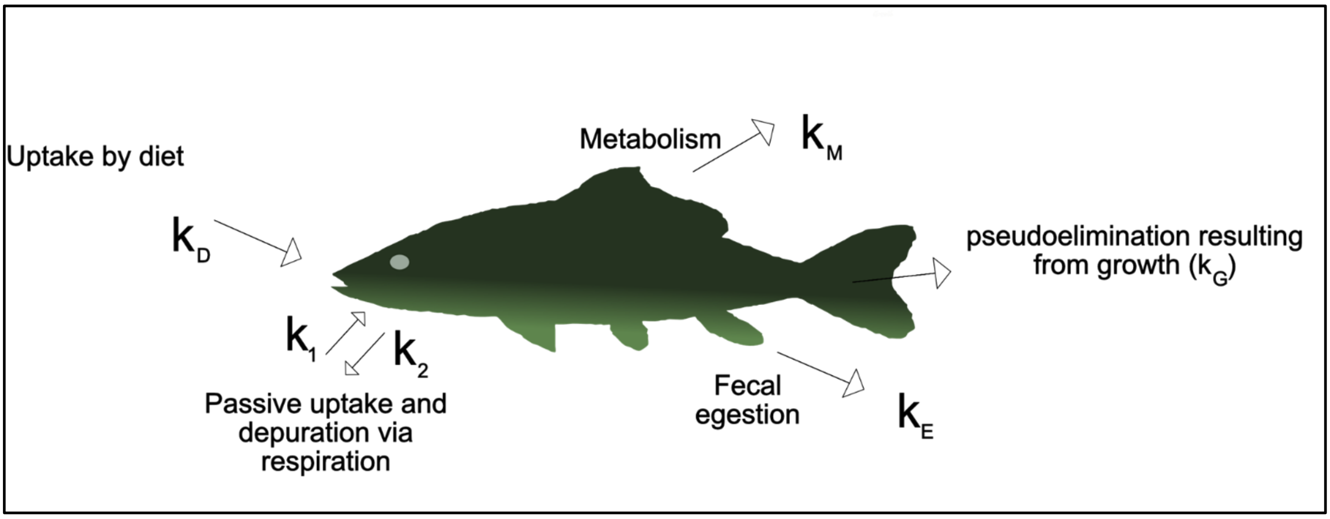
For hydrophobic organic chemicals the K_{bw} typically approximates K_{lipw}. Because octanol provides a good surrogate for lipids, relationships between K_{lipw} and K_{OW} have been proposed and used as a screening tool for bioaccumulation assessment (Mackay, 2001).

However, it is important to recall that uptake and accumulation are dynamic processes, and thermodynamic equilibrium between an organism and the surrounding environment may not be achieved (Mackay, 2001; Schwarzenbach et al., 2017). Indeed, because uptake and accumulation depend on several rate processes occurring at the same time, bioaccumulation assessment factors (BAFs) measured in the lab or in the field are known to change as a function of time (Arnot and Gobas, 2006). The key rates of uptake and elimination include uptake via respiration (k_1), dietary uptake (k_D), loss by respiration (k_2), excretion (k_E), metabolism (k_M), and growth (k_G). Consequently, the concentration of a chemical in the organism (C_{org}) at steady-state can be modelled as:

$$C_{org} = (k_1 \cdot C_w + k_D \cdot C_D) / (k_2 + k_M + k_E + k_G) \qquad \text{(Eq. 4)}$$

Where C_w and C_D represent the steady-state concentrations of the chemical in the aqueous system and the diet, respectively (Mackay, 2001; Arnot and Gobas, 2004). Figure 10 schematically illustrates the various key processes that influence the bioaccumulation of chemicals in fish, which include both passive and active uptake processes that result in the accumulation of hydrophobic chemicals within the lipid reservoirs of an organism. At steady-state, a BAF, defined as the ratio of the C_{org} and the bioavailable concentration in the test system, can be determined, and used to assess the bioaccumulation potential of a given chemical.

Figure 10: Schematic illustration representing some of the key processes influencing the bioaccumulation of a chemical in a fish. The various k values are assumed to be first-order rate constants and can vary depending on the biological traits of the organism

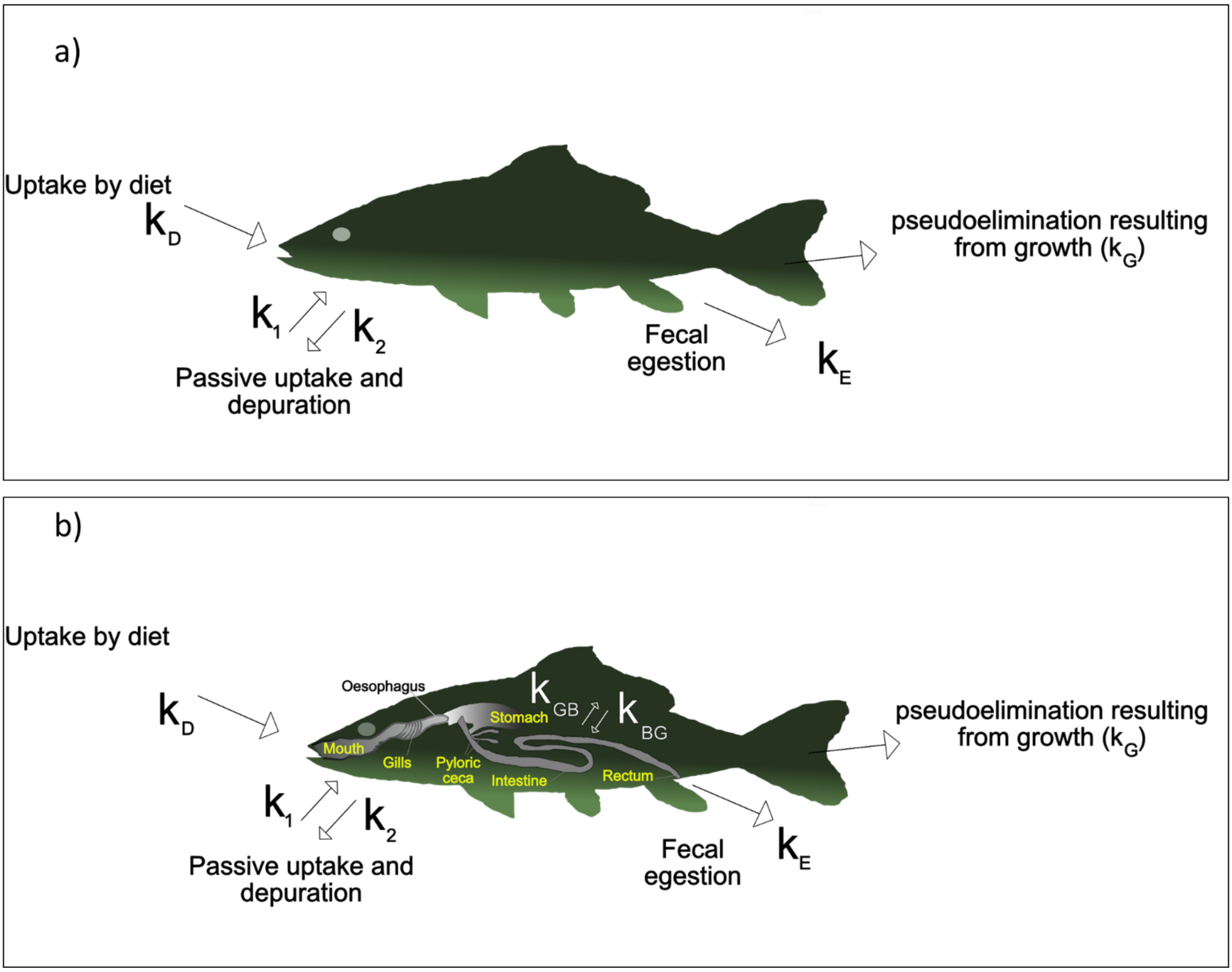


A significant amount of research has been undertaken throughout the last several decades that supports the mechanistic understanding of the bioaccumulation of chemicals, most of which is based on the processes described in the above equations and illustrated in Figure 10. The research that has been conducted has enabled the development and application of mass balance models for use within regulatory instruments, and has played an important role in screening and prioritising chemicals with respect to their bioaccumulation potential (Mackay et al., 2001; Arnot et al., 2006; Czub et al., 2008; Powell et al., 2009; Mansouri et al., 2012; Selck et al., 2012; Vestergren et al., 2013; Lombardo et al., 2014; Princz et al., 2014; Stieger et al., 2014). Mass balance models have also proved useful in estimating C_{org}, information that can then be used to extrapolate and strengthen the interpretation of OAE (McCarty et al., 1992; McCarty and Mackay, 1993; Van Wezel et al., 1996; Legierse et al., 1999; Escher and Hermens, 2002; Meador, 2006; Ashauer and Escher, 2010; Escher et al., 2011; McCarty et al., 2011; ECETOC, 2013; Armitage et al., 2014; Mackay et al., 2014; ECETOC, 2016; Fischer et al., 2017).

Analogous to the development and application of mass balance models to describe the biological uptake and accumulation of soluble chemicals, it would be advantageous to work towards the development of similar tools to characterise uptake and accumulation of sparingly soluble and particulate substances. Several reviews related to the biological uptake of NMs by aquatic organisms have been published, which summarise the results from various empirically-based lab studies (Klaine et al., 2008; Zhao et al., 2011; Hou et al., 2013; Peng et al., 2017; Bundschuh et al., 2018). Similarly, lab-based studies reporting the uptake of MP have also been reported (Ugolini et al., 2013; Setälä et al., 2014; Van Cauwenberghe et al., 2015; Vandermeersch et al., 2015; Watts et al., 2015; Grigorakis et al., 2017; Zhu et al., 2018). Observations of uptake vary widely, with little quantitative mechanistic insight identifying key processes influencing the potential of either MP or NMs to bioaccumulate, or distinguishing uptake/translocation into tissues from ingestion and/or simple presence in the gastrointestinal tract of the organism. Consequently, development of a systematic framework for standardised testing would enable greater mechanistic insight regarding biological uptake, and would further strengthen screening and prioritisation with respect to bioaccumulation potential of sparingly soluble substances and particles, such as MP and NMs (Hou et al., 2013; Vandermeersch et al., 2015; Bundschuh et al., 2018).

In this chapter we discuss several parameters that might represent key processes to include within a mechanistic uptake and accumulation model for particulates and sparingly soluble substances. To help stimulate discussion, the theoretical models that have been proposed by Lo et al (Lo et al., 2015) and Arnot and Mackay (Arnot and Mackay, 2018) to assess the bioaccumulation of hydrophobic organic chemicals via a dietary exposure scenario are adopted (Figure 11).

Figure 11: Two different conceptual models for particle uptake from water via respiration and/or adsorption to the surface of the organism (k_1) and the diet (k_D) into an aquatic organism and elimination to the water from respiration and/or desorption from the surface of the organism (k_2) and egestion (k_E). Model a) considers the organism to consist of one 'body' compartment. Model b) separates the lumen of the GIT and the rest of the body and includes a mechanism for particle transport between the two compartments (k_{GB} and k_{BG}).



Starting with the empirical observations that MP and NMs are accumulated within the whole organism, a relatively simple mass balance model can be formulated, which captures key input and output parameters (Figure 11a). However, to develop greater mechanistic understanding a three-compartment model, which would include the aqueous phase, the gastrointestinal tract (GIT), and the tissues of the fish provides a more realistic conceptual model related to biological uptake (Figure 11b). Quantitatively assessing the mechanisms of uptake across the epithelial tissues of the GIT are thus needed. Unlike hydrophobic organic chemicals, biological uptake of MP and NMs cannot be described by equilibrium partitioning theory, with other processes, such as phagocytosis, pinocytosis, and receptor-mediated endocytosis possibly influencing cellular uptake (Zhao et al., 2011; Praetorius et al., 2014). Indeed, considerable research within the biomedical community has been undertaken in an attempt to better understand the mechanistic uptake of NMs, which could aid in the delivery of active pharmaceutical ingredients; however, some of the most important basic aspects like the process of cellular uptake, metabolism, mechanism of cytotoxicity, intracellular location and translocation remain poorly understood (Zhao et al., 2011). Most studies of biological uptake of NMs and MP observe the particles largely passing through the GIT of the organism (Skjolding et al., 2016). Consequently, working towards an improved mechanistic understanding of both the physicochemical properties of the particles and their interactions with the physiological traits of aquatic organisms (such as with the gills, oesophagus, stomach, pyloric ceca, and the intestines) may lead to improved models for screening and prioritising their bioaccumulation potential.

4.2 Influence of physicochemical properties on uptake and accumulation

In Chapter 2 several physicochemical properties of sparingly soluble liquids and particles are highlighted, and have been shown to be important parameters influencing agglomeration and aggregation in an aquatic test system (Chapter 3). Key parameters include intrinsic properties, such as the chemical composition of the particle and surface chemistry, as well as extrinsic properties, such as particle shape and size. Intuitively, the physicochemical properties of particles will strongly influence biological uptake and accumulation. It is notable, however, that when considering the biological uptake of particles differences in uptake between agglomerates/aggregates and singly-dispersed particles can occur and can complicate interpretation of empirical observations. For instance, NMs that form large aggregates to form micro-sized particles, tend to settle out of solution, altering their bioavailability, and therefore their potential for biological uptake by pelagic species under aqueous exposure conditions (Klaine et al., 2008). On the other hand, Zhu et al (2010) observed significant uptake of aggregated NMs (median particle size about 3 µm) by daphnids, reporting concentration dependent bioconcentration factors (BCFs) of between 10^3 and 10^5 L/kg. The high BCF values reported for aggregated NMs in *Daphnia magna*, however, are likely due to physiological traits of the tested organism, which include the capability of filtering particles with sizes ranging from 0.4-40 µm, thus enabling a more efficient uptake of larger sized aggregates of NMs (Geller and Muller, 1981; Gophen and Geller, 1984; Filella et al., 2008; Hou et al., 2013). Knowledge of the individual particle and aggregate sizes can thus help interpret biological uptake. For instance, Rosenkranz et al (2009) observed uptake of 20 nm particles for *Daphnia magna*, representing a particle size below the filter mesh size for the organism. In this instance, uptake was suggested to have occurred passively as a result of ventilation or was due to the uptake of larger particle aggregates, a process which was not characterised by Rosenkranz et al (2009). Consequently, the particle size of individual particles and/or aggregates/agglomerates in relation to the feeding strategy of the organism and species is an important parameter to consider, particularly the influence of particle size and physiological and behavioural traits on bioavailability (Lead et al., 2018).

Once ingested by an organism, the size and shape of particles has also been observed to influence cellular uptake. For instance, the cellular uptake of Herceptin-colloidal gold nanoparticles (2-100 nm) has been shown to be most efficient in the size range of 40-50 nm, due to the direct balance between the multivalent crosslinking of membrane receptors and the process of membrane wrapping involved in receptor-mediated endocytosis (Jiang et al., 2008b; Verma and Stellacci, 2010; Zhao et al., 2011). Particle shape, on the other hand, can influence the time for membrane wrapping involved in endocytosis, with spherical NMs entering cells more easily than rod-shaped particles (Jiang et al., 2008b; Qiu et al., 2010). The process of cellular uptake for aquatic organisms is thus confounded by the bioavailability and uptake of particles within a specific size range and shape that lend themselves well to endocytosis and will likely represent an important contribution towards characterising k_{GB} and K_{BG}, as shown in Figure 11b. Given several studies that report translocation of MP within organisms (Browne et al., 2008; Rosenkranz et al., 2009; Zhu et al., 2010; Farrell and Nelson, 2013; Della Torre et al., 2014; Lu et al., 2016; Pitt et al., 2018), coupled with those studies that provide contradictory observations that do not observe uptake into biological tissues (Petersen et al., 2009; Hou et al., 2013; Ugolini et al., 2013; Kaposi et al., 2014; Setälä et al., 2014; Watts et al., 2014; Grigorakis et al., 2017; Skjolding et al., 2017; Dawson et al., 2018; Redondo-Hasselerharm et al., 2018), greater understanding is clearly needed to clarify the mechanisms that might influence the relative importance of translocation and accumulation of particles within the tissues of aquatic organisms (Lusher et al., 2013).

Characterisation of factors that might influence k_1, as shown in Figure 11b, should thus be prioritised. As is discussed further below (Section 4.3), the physiological traits of the organism will strongly influence feeding behaviour, with the mesh size of aquatic filter feeders needing to be well defined. However, biological uptake by non-filter feeders has also been studied, whereby ventilation has been suggested as an important parameter to include within the construction of a mechanistic model to describe the movement and retention of MP in the gut and gills of the shore crab, *Carcinus maenas* (Watts et al., 2014). In their development of a mechanistic model to assess the movement of particles through the shore crab, Watts et al (2014) include mechanisms to account for the adsorption of particles to the surface of organs, such as the lamellae within the gills of the organism, as well as within the gut. Adsorption in this case is modelled as a reversible process, with rates for adsorption and desorption included (Watts et al., 2014). The structure of the model proposed by Watts et al (2014) is thus consistent with the conceptual model illustrated in Figure 11b and provides an excellent illustrative example for how such models could be further developed.

With the development of mechanistic models aimed at strengthening the characterisation of k_1, sensitivity analysis could then be performed that would help to screen and prioritise key properties of particles that facilitate their uptake by organisms. Coupled with the development of species-specific models that account for differences in physiological traits, both the key properties of particles and most sensitive species could thus be identified. Based on several observations from existing empirical studies examining the uptake of both NMs and MP, there appears to be consensus that size and shape of the particles represent important properties (as discussed above). However, adsorption to the external surface of an organism and/or internal adsorption appears to be a mechanism that is not only influenced based on size and shape, but which would also include an influence of the surface charge of the particle. For instance, Geitner et al (2016) developed a bioassay to quantify an attachment efficiency of nanoparticles with varying surface properties, including both negatively and positively charged modified gold nanoparticles (coated with citrate and poly(allyamine hydrochloride), respectively) as well as an environmentally relevant negatively charged material with greater hydrophobicity (gold nanoparticle coated with Suwanee River humic acid), to both algal cells and *Daphnia*. The results observed generally report a higher attachment efficiency for the positively charged particles. Building on the data obtained from their bioassays, Geitner et al (2016) also include a suggestion for the application of a relatively simple model, which applies the holistic surface affinity property they characterise, within a simple food web to estimate at which values particle surface affinity might lead to trophic bioaccumulation. The approach presented by Geitner et al (2016) thus attempts to simplify the complex interactions that might occur between the various intrinsic and extrinsic properties of particles and system dependent properties to facilitate screening and prioritisation estimates for potential of particles to bioaccumulate. Ideally, a tiered process that utilises a combination of simple models, such as that proposed by Geitner et al (2016), combined with more sophisticated mechanistic models, such as the conceptual model shown in Figure 11b, will strengthen our overall understanding of the complex interactions that can occur between aquatic organisms and particles, both natural and artificial in origin.

4.3 Physiological traits

As discussed above, the physicochemical properties of size, shape, and the chemistry of the surface of particles, represent properties that influence biological uptake and accumulation of particles. However, the interactions between aquatic organisms and suspended solid particulates have evolved over millennia, with the physiological traits of an organism thus representing a critical component towards assessing uptake, accumulation, and interpreting OAEs. Physiological traits are intrinsic properties of an organism, and include physiological processes such as phenotypic plasticity, metabolism, feeding strategy, and ventilation (Killen et al., 2017). In this section, those processes that influence the uptake of particles are emphasised; however, it is notable that traits, particularly phenotypic plasticity, are important to consider when interpreting OAEs. For example, gills can exhibit significant plasticity in response to hypoxia, temperature, and high turbidity, which allows an organism to maximise oxygen uptake while limiting absorption of toxic substances (Sollid and Nilsson, 2006; Nilsson et al., 2012; Hess et al., 2015; Killen et al., 2017). Plasticity in this context can thus refer to the capacity of an organism to respond and adapt to an environmental stressor, resulting in some species being more sensitive than others depending on their relative phenotypic plasticity. The temporal and spatial variability associated with changes that an organism may have to an exposure of an environmental stressor contrasts the conceptual model shown in Figure 11, which assumes steady-state conditions. Incorporating capability to address dynamic fluctuations in stressor exposure, as well as physiological traits that enable an organism to respond and adapt, are characteristics of higher-tier models that might be developed.

The ingestion of particles of varying sizes for aquatic organisms differs from species to species, and intuitively is related to the size of the organism itself and the feeding strategy of the species. There are numerous studies that have investigated the feeding behaviour of aquatic organisms in relation to particle uptake (see for instance Ward and Shumway (2004) and Riisgård and Larsen (2010)). Some organisms are selective with respect to the ingestion of particles, whereas others are non-discriminating, and consume a wide range of shapes and sizes. Further complicating the process are the hydrodynamics of the system, which is an important factor influencing the dispersibility of particles within a test system, and therefore the interactions between the particles and the organism. In a detailed review summarising the various mechanisms influencing the uptake of particles by suspension-feeding invertebrates, Riisgård and Larsen (2010) demonstrate that, despite the complexity in particle uptake mechanisms, there are two main processes: (1) Filtering or sieving, which is found in passive suspension feeders that rely on the movement of the water to bring particles to them; and (2) paddle-like flow manipulating systems that redirect approaching suspended particles to the organism. In the case of suspension-feeding invertebrates, which are an important group of organisms at the base of the food web, and include bivalves, polychaetes, ascidians, bryozoans, crustaceans, sponges, and cnidarians, the particle size range they are capable of ingesting is described as typically ranging between 2 to

200 µm but can also include free-living bacteria which exist in the size range of 0.5 to 2 µm (Riisgård and Larsen, 2010). For these organisms it is likely that exposure to particles and/or particle aggregates within the size range of about 0.5 to 200 µm will be dominated by direct ingestion, depending on the selectivity of the organisms in discriminating differences between different types of particles. As a specific example, *Daphnia*, which are an important model species used in assessing ecotoxicological effects to chemical stressors, consume particles from around 1 µm up to 50 µm, although particles of up to 70 µm in diameter have been observed in the gut content of larger individuals (Ebert, 2005). Because of the ecological significance of *Daphnia* in freshwater food webs, their filter-feeding behaviour has been intensely studied. Of interest are those studies that measure and model the appendage beat rate in relation to changes in relative concentrations of food (Porter et al., 1982; Plath, 1998; Kmeť and Straškraba, 2004). The area of study remains somewhat contested, but it is suggested that insight that has been gained with respect to the foraging strategy of filter feeders be included in the development and interpretation of ecotoxicity studies using these organisms in assessing uptake and effects of particulates, such as NMs and MPs.

For instance, in a recent study published by Vinther et al (2014), the relationship between the filter mesh spacing and the food items consumed by suspension-feeders is further illustrated. The relationship presented by Vinther et al (2014) suggests a statistically significant power relationship between filter mesh spacing and the lower and upper limits of the particle (i.e. prey) sizes they typically ingest, with the upper particle size estimated as 11.772(*w*)0.89 (R^2 = 0.87) and the lower size estimated as 1.445(*w*)1.01 (R^2 = 0.92), where *w* refers to the filter mesh spacing of the suspension-feeding organism. This observation, which includes suspension-feeding invertebrates, fish (anchovy, Rainbow trout, whale shark), mammals (Bowhead whale), and birds (flamingo), potentially provides a strategy for targeted research aimed at assessing which organisms are most susceptible to ingesting particles and/or particle aggregates within a certain size range, as well as possibly providing insight regarding the ability to estimate the trophic transfer of particles.

When exposed to MP, the filter feeding strategy of copepods, euphausids, and doliolids is reported to be dominated by the filter-feeding behaviour of these organisms, with differences in how the organisms manipulate the particles to ingest them being further described (Cole et al., 2015). Given the biological variance associated with both physiological and behavioural traits, it is thus likely that the uptake term k_1 illustrated in Figure 11 will require species-specific details. Lastly, a curious observation reported in several studies, is a concentration dependent ingestion rate, with ingestion rates of MP increasing with increasing concentrations (Kaposi et al., 2014; Au et al., 2015; Mazurais et al., 2015). Under environmentally relevant conditions, where exposure to MP and NMs is known to be orders of magnitude lower than in most lab-based studies (Phuong et al., 2016), the implication would result in relatively low ingestion rates for aquatic species in the environment. Indeed, based on results reporting MP in the GIT of a large variety of fish species, concentrations are reported as averaging about <2 particles/individual (Boerger et al., 2010; Foekema et al., 2013; Lusher et al., 2013; NIVA, 2014).

Once ingested by aquatic organisms, the residence time within the GIT represents another important factor to include in advancing our mechanistic understanding of the uptake and accumulation of MP and NMs. In several studies that have investigated the uptake and egestion of MP and NM particles, complete egestion is typically reported once exposure is removed (Au et al., 2015; Grigorakis et al., 2017; Dawson et al., 2018). The rate at which particles are eliminated by the egestion of faecal matter can thus be expressed by the faecal elimination rate constant. For invertebrates, such as *Daphnia*, egestion can be on the order of minutes, with

GIT residence times typically increasing for larger organisms. In their study aimed at characterising the retention of MP relative to the egestion of the natural diet of fish, Grigorakis et al (2017) fit their data to an exponential model using non-linear least squares according to:

$$\%\text{Retained} = 100 \cdot e^{-B \cdot \text{time}} \qquad \text{(Eq. 5)}$$

Where 100 is a constant forcing 100% of gut contents retention at time 0, *B* is the fitted coefficient, and time is the amount of time since feeding (h). The General Linear Model (GLM) they fitted resulted in a coefficient of 0.069, with the time to evacuate 50% and 90% of GIT contents being 10 and 33 h, respectively (Grigorakis et al., 2017). Recognising that GIT residence time will vary across the different life cycles of a species and across species, application of the approach taken by Grigorakis et al (2017) in any study aimed at investigating uptake of particulates, such as MP and NMs, could thus prove useful in parameterising k_E (Figure 11), and strengthen both our overall understanding of bioaccumulation potential and interpretation of OAEs.

It is perhaps curious to note that many studies report differences in egestion rates of particles, whereby the ingestion of natural food appears to facilitate particle transport along the GIT. In the absence of food, non-natural particles are reported as taking significantly longer to egest. In some aquatic toxicity test systems, the feeding protocol for the test may consequently influence the GIT residence of the particles, which could result in observations of accumulation and OAEs that may be artefacts of the test method and may not appropriately represent ecologically relevant behaviours. For instance, standard protocol for aquatic acute toxicity testing does not include any feeding, which will consequently influence the GIT residence of the particles. It is recommended that appropriate controls be included within test design to appropriately account for such artefacts and attempt to interpret results accordingly. For instance, translocation of MP and NMs may be related to longer GIT residence times, which provide increased potential for endocytosis. Consequently, the relative magnitude of k_E may influence k_{GB}, and should be more thoroughly investigated to strengthen overall mechanistic understanding.

As previously discussed, Figure 11 represents a conceptual model related to key parameters needed in estimating particle uptake and accumulation within an aquatic organism. In this section, details of some of the key physiological traits that might strengthen the parameterisation of mechanistic models are explored; however, it is suggested that organism behavioural traits and influence of differences between species-specific life cycle stages also be considered when further progressing the parameterisation of mechanistic models.

4.4 Dietary uptake and trophic transfer

There is considerable evidence, first reported some years ago (Carpenter et al., 1972), that MP could be mistaken for food sources in the aquatic environment. More recently this has received substantial attention within the scientific community. A potentially important, but poorly understood, component surrounding the ingestion of MP is the potential for the particles themselves to be transferred through the food web and elicit harmful effects on higher trophic organisms. A key area of interest is thus directed towards assessing how MP compare with algal cells, which represent the base of the food web. Fendall and Sewell (2009), for example, concluded that the range of MP found in cosmetic ingredients resemble algal cells in terms of their size and shape, implying that browsers that feed on algae are likely to consume MP of a similar size range. Furthermore, Doyle et al (2011) examined algal communities within a natural water column, and detected MP derived from product fragments, fishing net and line fibres, and industrial pellets, confirming the potential for organisms to consume MP while feeding on algae (Moore et al., 2001; Lattin et al., 2004; Collignon et al., 2012).

Given the potential for particles to be consumed along with organisms at the base of the food web, the question arises as to whether MP and NMs could be biomagnified along a food web, analogous to the bioaccumulation and biomagnification of persistent organic pollutants, which involves a concentration step that occurs between trophic levels. For instance, organisms that feed on algae may consume relatively small numbers of MP that then have the potential to translocate from the GIT into tissues and accumulate in their system; organisms that feed on them can then encounter both direct and indirect dietary exposure, increasing their overall exposure that might lead to an additional quantity of particles being accumulated (Wright et al., 2013). While it has been speculated that this process occurs, there are only a limited number of studies that have attempted to quantify and characterise the potential for trophic transfer of MP (McMahon et al., 1999; Ericksson and Burton, 2003; Murray and Cowie, 2011; Choy and Drazen, 2013; Farrell and Nelson, 2013; Setälä et al., 2014; Eerkes-Medrano et al., 2015; Abbasi et al., 2018; Bonanno and Orlando-Bonaca, 2018; Zhu et al., 2018). Recent studies that have investigated the trophic transfer of MP through the aquatic food web have primarily been based on controlled laboratory studies, which attempt to investigate the relative importance of indirect ingestion of MP on predatory species. For instance, having already detected MP fibres in the GIT of Norwegian lobsters during a field investigation, Murray and Cowie (2011) fed ‘seeded’ fish (which had MP artificially placed inside them) to lobsters. They observed that 100% of Lobsters had ingested the seeded MP, demonstrating that MP fibres can be transferred from fish to predatory crustaceans in the marine environment. Although trophic transfer occurred, there is no concentration difference between the trophic levels, and therefore no apparent bio-‘magnification’.

A 2013 study by Farrel and Nelson (2013) investigated the transfer of 0.5 µm polystyrene MP from mussels (*Mytilus edulis*) to crabs (*Carcinus maenas*). The maximum amount of MP that could be observed in crabs haemolymph was 0.04% of the amount to which the mussels were exposed, indicating that a very small number of MP had been transferred to the crabs. MP were also found in the stomach, hepatopancreas, ovary and gills of the crabs in decreasing numbers over the trial period, suggesting that MP were effectively eliminated by the crabs as a function of time (Farrell and Nelson, 2013). In another study, the food web transfer of 10 µm polystyrene MP from zooplankton to Mysid Shrimp was investigated (Setälä et al., 2014). Microscopic observations of mysid intestines showed the presence of zooplankton prey and microspheres after 3 hour incubation, indicating that MP had been transferred (Setälä et al., 2014).

Overall, the available evidence suggests that certain MP types may be transferred from organisms at various food web levels. The extent to which this occurs will be strongly influenced by particle concentration, particle size, and particle shape in relation to the feeding strategy of the organisms in the food web. The significant variability that can exist in the various parameters associated with the physicochemical properties of the particles coupled with variance in physiological and behavioural traits, complicates the parameterisation of k_D shown in Figure 11. Unlike hydrophobic organic chemicals, for which bioaccumulation is thermodynamically favoured (Macdonald et al., 2002), and leads to significant biomagnification in the concentration of chemicals moving to higher trophic levels, the accumulation of particles is not thermodynamically defined (Praetorius et al., 2014). Consequently, there is not the same energetically favoured process that would lead to increasing concentrations of particles in organisms at higher trophic levels as there is for hydrophobic chemicals.

Nevertheless, both dietary and non-dietary exposure of particles can clearly occur. A challenge is perhaps in better understanding mechanistic relationships between particle size, ingestion, and egestion. For instance, based on the data presented by Vinther et al (2014), suspension-feeding invertebrates, such as cladoceran, mysid, and krill, typically prey on organisms <100 µm, and include eukaryotic algae and bacteria. As part of their feeding strategies, these organisms use either passive or active mechanisms to consume particles. In studies that have assessed the trophic transfer of particles by suspension-feeding invertebrates, particles <10µm have been observed to be efficiently egested as the particles move through the food web (Setälä et al., 2014). Consequently, while ingestion of particles of varying sizes occurs at all trophic levels, the efficiency of larger organisms to eliminate smaller particles appears to be substantive. Indeed, in their investigations into the presence of MP in seven common North Sea fish, Foekema et al (2013) did not observe the presence of any MP <300 µm, implying limited potential for particles <300 µm to accumulate as they move up the food web. This is also consistent with results reported by Choy and Drazen (2013), who do not report any particles <4mm in pelagic predatory fish collected in the central North Pacific. Consequently, it is suggested that the relationship between particle size and the potential for trophic transfer towards developing an improved understanding of impacts on organisms at different levels within a specific food web can be more closely linked to one another.

It is notable that environmental monitoring studies examining the presence of MP in the marine environment typically utilise nets, such as a manta trawl net, which has a mesh size of about 335 µm. When considering the varying sizes of organisms within a marine trophic food web, micro-organisms, such as zooplankton, feed on organisms <100 µm in size. If these organisms are consuming particles <100 µm, it seems reasonable to conclude that the mesh size of the sampling net is failing to adequately sample these pollutants. An alternative approach could possibly be based on the use of biomonitors, whereby organisms at the low end of the food web are used to assess the bioavailability of particles <100 µm in the aquatic environment. This information might strengthen our overall understanding of the environmental impact of MP and NMs on organisms at the base of the food web under ecologically relevant conditions. Given the efficiency of larger organisms to eliminate particles <100 µm, it is perhaps not surprising to find few studies, if any, that have observed the transfer of MP and NMs within an aquatic food web.

4.5 Conclusion

Whereas there exists significant research observing the biological uptake and accumulation of MP and NMs, results can often be contradictory. Inherent variability associated with the physiological and behavioural traits of organisms perhaps complicates the potential to mechanistically characterise the relationship between the intrinsic properties of particles and their potential to bioaccumulate. Nevertheless, some consistent themes do appear to emerge, whereby the size, shape, and surface charge of particles and particle aggregates represent important physicochemical properties to better understand. For instance, particles of about 50 nm in size have been shown to have the potential to translocate via endocytosis (Jiang et al., 2008b; Verma and Stellacci, 2010; Zhao et al., 2011), however, there are physiological traits of an organism which may effectively limit the exposure to particles above and below a certain size range that may further limit translocation. Particle concentration can influence the rate of ingestion; however, egestion rates remain relatively constant, with enhanced egestion of particles occurring as organisms feed.

5. OBSERVED ADVERSE EFFECTS

5.1 Introduction

As acknowledged throughout this report, insoluble particulates and poorly soluble liquids have the potential to adversely affect aquatic organisms. As a general observation, most aquatic toxicity studies investigating these materials typically provide limited insight regarding the mechanistic toxicological mode-of-action related to the OAE. In Chapter 2, and summarised by Equation 1, i.e. OAE = $\Sigma E_{iT} + \Sigma E_{Pint}$, adverse effects observed in aquatic toxicity tests are suggested to represent either the influence of an intrinsic toxicity or could be due to direct or indirect physical effects, or a combination of both.

Intrinsic toxicity, E_{iT}, is based on the relationship between the intrinsic physicochemical properties of a substance or mixture that can result in a sub-organism level adverse effect at a biochemical or cellular level of organisation and at a specific exposure concentration to negatively impact an apical endpoint. Intrinsic physicochemical properties of a poorly soluble liquid include its molecular structure, solubility in water (S_w), K_{ow}, adsorption coefficient (K_d), and its vapour pressure (P). For solid insoluble particulates, properties that may contribute to adverse effects also include the physical attributes of the particles, such as size, shape, and crystalline structure (See for instance Chapter 2).

The reporting of an OAE can be characterised by its EC_{50} (i.e. the concentration at which there is a 50% effect) and the slope of the concentration-response relationship, observed for a given apical endpoint (such as survival, growth, reproduction, etc.) (ECETOC, 2002). For most soluble chemicals the relationship between a specific toxicological mode-of-action occurring at the molecular/cellular level and the intrinsic properties of the chemical cause the OAE to be dominated by E_{iT}. However, for insoluble particulates and sparingly soluble liquids, E_{Pint} can strongly influence the OAE. Physical effects from interactions with particulates include various mechanisms (discussed below), such as blocking of fish gill membranes, encapsulation/entrapment of aquatic invertebrates, and a reduction of light intensity in algal tests (OECD, 2000). Furthermore, the pattern of responses due to E_{Pint}, whether from direct or indirect interaction of the particulate substance with the organism, tend to be less reproducible in comparison with results obtained for chemicals where the OAE is dominated by E_{iT}, and consequently do not lend themselves well to standard dose-response principles (Petersen et al., 2015; Jemec et al., 2016). Consequently, it is important to discriminate between E_{iT} and E_{Pint} when interpreting OAE involving insoluble particulates or sparingly soluble liquids, as this information is critical in helping to guide the risk assessment process.

Consistent with the overview provided in Chapter 3, laboratory *in vivo* ecotoxicity studies are designed to control the exposure route (e.g. waterborne, dietary, sediment, injection). In the natural environment, aquatic species are often simultaneously exposed to chemical substances through their diet, the water column and/or interaction with chemical substances in the sediment. The physicochemical properties of chemicals, their interaction with their environment, as well as the biology of aquatic organisms determine the potential for chemical exposure to aquatic life. Exposure within an aquatic toxicity test in the laboratory can be effectively controlled to simulate worst-case exposure conditions i.e. maximise bioavailability. Laboratory toxicity testing can include delivering the test chemical via direct injection to the organism, the diet, the sediment, or the water column. Direct injection into the organism may be done to better elucidate the chemical toxicological mode-of-action; however, it is not reflective of a ‘true’ environmental exposure condition; thus, this method of delivery is generally not recommended for aquatic toxicity studies (Harris et al., 2014). Dietary and/or sediment exposure can be employed with insoluble and/or hydrophobic substances, especially if the exposure route is relevant in the environment and exposure via the water column is logistically not possible (Harris et al., 2014).

Generally, the dissolved fraction of a substance is the fraction that is considered most bioavailable for uptake by aquatic organisms, and chemicals that are exposed via the water column are absorbed, for example, across the gills and can enter the circulation directly with no possibility of first-pass metabolism (Rand et al., 1995; Weyman et al., 2012). Thus, the dissolved fraction of a chemical is generally considered to be responsible for influencing the ‘true’ aquatic toxicity (Weyman et al., 2012). In aquatic exposure studies, chemicals are generally tested up to a maximal concentration of 100 mg/L, which sets the upper limit for environmentally relevant toxicity and classification and labelling purposes. Chemicals with water solubility below 100 mg/L need to be tested up to their solubility limit, at which point saturation of the chemical within the test system is attained. The OECD’s Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures is an invaluable resource when determining the best approach for testing an insoluble chemical (OECD, 2000).

Over the last 10-20 years, several peer-reviewed publications have reported OAE associated with particulates, such as with NMs and MP, and have been summarised in several recent reviews (Hu et al., 2016a; Skjolding et al., 2016; Connors et al., 2017; Hjorth et al., 2017). Based on their critical review of the published literature, a series of recommendations for conducting ecotoxicity tests with insoluble substances have been developed by researchers (Petersen et al., 2015; Connors et al., 2017). Petersen et al (2015), for instance, provide guidance for preparation of dispersions, dose metrics, as well as maintenance and measurement of NMs in laboratory studies. Connors et al (2017), on the other hand, recently reviewed the available toxicity studies involving MP and offered nine guiding principles to improve hazard assessments with these materials. Finally, building on the learnings of NM research, Hüffer et al (2017) provide constructive suggestions that could be applied to toxicity studies involving MP.

An important consideration to note when performing an aquatic toxicity test conducted with particulates, such as NMs and MP, relates to the potential for dissolution of metal ions from NMs and/or the leaching of monomers, additives, and other chemical contaminants that might be associated with a polymer coating of a NM or released from MP (Lead et al., 2018). The potential release of chemical contaminants into a toxicity test system introduces the possibility of an E_{iT} that may be inherent to the freely dissolved chemical contaminant fraction, and which may subsequently complicate interpretation of an OAE associated with the testing of the particle itself. In these instances, both fractions can result in adverse effects, with both E_{iT} and E_{Pint} influencing the OAE. Separating each component of the OAE is thus challenging where both the dissolved and particulate fractions combine to influence the exposure scenario. To adequately address the concerns described, the adoption of appropriate quality control and quality assurance methods should be employed, such as experiments aimed at analytically characterising the material to be tested, quantifying the monomer residue levels and/or the fraction of additives that may leach from the particles, and quantification of the dissolution rate of the metal ion for NMs. Furthermore, methods to reduce effects associated with chemical contaminants should be developed and adopted as well as the inclusion of appropriate test-study controls and reference substances, necessary for strengthening the interpretation of study results.

While there are unique properties and test design challenges to consider when determining the potential hazards associated with exposure to insoluble particulate and sparingly soluble substances in aquatic environments, the protection goal of maintaining healthy populations, communities, and ecosystems in the environment remains the same. Apical endpoints, such as survival, growth, reproduction, and development have been adopted in standardised ecotoxicity test guidelines, because these parameters are known to be predictive of population-level effects (U.S. Environmental Protection Agency (EPA), 1998; EC, 2002). Non-standard or sub-organismal endpoints that measure effects at the biochemical or tissue level of organisation may be useful, e.g. as indicators of exposure or for understanding the toxicological mode-of-action of a substance, however these endpoints usually lack a clear connection to adverse effects and may instead be reflective of adaptive responses of the organism to the external environment (Nichols et al., 2011). Where elucidating mechanistic understanding of OAEs is critical, data based on non-standard or sub-organismal endpoints can therefore represent a confounding factor in differentiating between E_{iT} and E_{Pint}.

When interpreting the effects of toxicity studies, it is important to understand the toxicological mode-of-action that is driving the OAE. Understanding the toxicological mode-of-action can aid in interpreting the toxic response that might occur under environmentally relevant exposure scenarios. Additionally, mechanistic understanding of the toxicological mode-of-action can help to inform the potential interactions with regard to mixtures of chemicals in the environment. Remediation and mitigation of risk can also be best undertaken when the toxicological mode-of-action is well understood. As discussed above and elsewhere in this report, the appropriate interpretation of an OAE associated with exposure to insoluble particulates and sparingly soluble substances can be particularly challenging to decipher. This chapter summarises the current state-of-science underpinning our understanding of processes that influence an E_{Pint} associated with exposure to insoluble particulates and sparingly soluble substances, and which largely dominates the OAE reported in the literature for these materials.

5.2 Direct and indirect processes influencing E_{Pint} for particulates

Physical effects in exposed organisms are due to non-specific, mechanical perturbations that are not linked to specific chemical reactions. Direct and indirect physical effects due to exposure to insoluble particulate substances have been observed among various aquatic organisms representing different trophic levels. Direct physical injury is the result of direct interaction of the insoluble particulate with the exposed organism, and indirect physical effects occur when the insoluble particulate interacts with environmental media or other components of the environment to cause adverse outcomes to organisms in that same environment. The physical effects from exposure to insoluble particulate substances occur in patterns that are not reflective of the classical dose-response relationships observed for soluble, non-particulate chemicals. For example, Jemec et al (2016) exposed *Daphnia magna* to microfibers in a standard acute toxicity test design, and daphnids were either pre-fed or not with algae prior to the exposure. Increased mortality was primarily observed among *Daphnia magna* exposed to the microfibers without pre-feeding, but there was no direct connection between the fullness of the gut with microfibers and observed mortality (Jemec et al., 2016). Thus, the mortality of *Daphnia* was not dependent on the exposure dose and varied significantly between different replicates of the same exposure concentrations and regime. Due to the variability in observed effects an EC_{50} value could not be determined (Jemec et al., 2016). It is likely that the large variability in response between replicates of the same treatment level was due to the random feeding pattern and heterogeneous exposure conditions of the aqueous dispersion of microfiber particles. The variability and dose-response are not typical of results obtained from aquatic toxicity tests for soluble test materials, which are homogeneously distributed in the test media (Jemec et al., 2016). The pattern of variability reported by Jemec et al (2016), however, is commonly observed in aquatic toxicity tests that include particulates and has long been recognised to be a confounding factor in aquatic toxicity studies.

Lastly, it should be noted that a common observation amongst aquatic toxicity tests involving particulates, such as NMs and MP, are the relatively high concentrations that are reported to cause physical effects in laboratory studies with aquatic organisms, which are typically reported as being several orders of magnitude greater than the concentrations expected in the environment (Besseling et al., 2014). Consequently, extrapolation of laboratory-based OAE may not necessarily provide relevant information in helping to inform an environmental risk assessment. Other confounding factors associated with laboratory-based exposures include limited insight regarding the potential physical transformation of the particles, such as might occur within a waste-water treatment system, interactions with natural organic matter and the formation of hetero-aggregates, and/or alterations of the surface chemistry of the particles, which might include the formation of a biofilm, and how these processes might alter the exposure scenario for aquatic organisms. Thus, the altered physical properties of particles in the natural environment can change how they may interact with biological receptors in the lab vs. field (Baun et al., 2008; Batley et al., 2013).

5.2.1 E_{Pint} in aquatic plants

With respect to plant and algal species, several examples of physical effects from insoluble particles have been observed and reported. For instance, iron oxide NMs have been observed to physically cover the surface of algal cells with agglomerates, resulting in decreased size of the microalgae (Demir et al., 2015). Alternatively, carbon NMs can directly interact with plants to block stomata, resulting in foliar heating and altered physiological processes (Basiuk et al., 2011). In an algal toxicity study with positively and negatively charged nano-sized polystyrene spheres (20 nm), it was observed that both types of nanoparticles adsorbed onto microscopic freshwater and marine green algae (Bhattacharya et al., 2010). The mechanism of adsorption was attributed to hydrogen bond formation between aromatic structures of the cellulose and the polystyrene nanoparticles. Positively charged nanoparticles were observed to adsorb more than the negatively charged nanoparticles due to the electrostatic attraction between the carboxyl and sulphate groups in the cellulose of the algal cell wall (Bhattacharya et al., 2010). The researchers indicated that the direct physical adsorption of these plastic beads hindered algal photosynthesis possibly through direct physical blockage of light and gas exchange by the nanoparticles (Bhattacharya et al., 2010).

In a similar algal toxicity study, neutral and positively charged nanoplastic particles (20-500 nm) were observed to adsorb to the cell wall of microscopic green algae (*Pseudokirchneriella subcapitata*) due to the slightly anionic surface of the algal cell wall which contains carboxylic acid residues (Nolte et al., 2017). However, due to the relatively low levels of observed direct adsorption of the nanoparticles to the cell surface, the observed algal growth inhibition was suggested to be unlikely to be due to direct physical injury but was more likely to be the result of indirect effects via light scattering of free particles and agglomerates in the media. Furthermore, the release of multivalent ions resulting in nutrient depletion or increased osmotic pressure was also a possible indirect mechanism to explain observed growth inhibition in this study (Nolte et al., 2017). In another growth inhibition study with *Skeletonema costatum*, a marine microalga, it was observed that smaller MP (average diameter of 1 µm) were able to cause growth inhibition, whereas larger MP pieces (average diameter of 1 mm) did not result in growth inhibition (Zhang et al., 2017). In this case, an indirect shading effect was evaluated by using a special test vessel with the upper portion being light-proof and housing algal cells, while the lower portion housing the MP was completely transparent to allow a pathway for light to travel to the algal cells. In this study, the shading properties of both the large and small MP was insufficient to inhibit algal growth, whereas growth inhibition was observed when the smaller microparticles were housed with the marine algae (Zhang et al., 2017). Direct interaction of the small microparticles (average diameter of 1 µm) was observed using scanning electron microscopy, by which the interaction of MP with caveolaes (lipid raft invaginations in cell membranes that are rich in lipids and proteins and play important roles in signal transductions) was reported, thus limiting nutrition, light, and CO_2 and O_2 exchange between the test media and algal cells (Zhang et al., 2017). Direct interaction with the algae decreased with time, perhaps in response to increased agglomeration of the microparticles (Zhang et al., 2017). The inverse relationship between effect level and exposure time highlights the difference between toxic and physical modes of action and is an important consideration for environmental risk assessment.

5.2.2 E_{Pint} in aquatic invertebrates

The effects of both NMs and MP have been evaluated in several different invertebrate taxa, and direct and indirect effects have been observed (Hu et al., 2016a; Skjolding et al., 2016; Connors et al., 2017; Hjorth et al., 2017). In one study, silver nanoparticles were ingested by *Daphnia magna* following 48-hr acute exposure, and the particles accumulated under the carapace of the daphnids and caused abnormal swimming (Asghari et al., 2012). Based on this observation, silver nanoparticles were categorised as Acute 1 according to the Globally Harmonised System of Classification and Labelling (GHS). Reduced moulting for *Daphnia magna* was also observed at 0.001 mg/L and higher concentrations, but there was no impact on size (Gaiser et al., 2011). Nanosized (~100 nm) titanium dioxide exposure at 2 mg/L over a total test duration of 96 hours, resulted in 90% mortality among *D. magna* (Dabrunz et al., 2011). Based on the observed biological surface coating and subsequent reduced moulting rate and high mortality, moulting disruption of *D. magna* was suspected as causative in the adverse response (Dabrunz et al., 2011). Larger sized titanium dioxide particles (~200 nm) appeared less adverse than the smaller ~100 nm particles in this study (Dabrunz et al., 2011). Iron oxide nanoparticles coated with humic acids were found to clog up the filter apparatus and adhere to the exoskeleton of *D. magna* resulting in disruption of swimming and moulting activities, causing immobilisation and death of the organisms at concentrations ≥943 mg/L (Gonzalez-Andres et al., 2017). These examples indicate direct physical injury to the pelagic *D. magna* from elevated exposures to nano-sized insoluble particulates that directly interfere with mobility and moulting activities.

Direct physical injury of particulates have also been observed for *Daphnia longispina* when exposed to particulate iron, as well as the chemically-inert china clay (Randall et al., 1999). In both cases, chronic exposure over 21-days resulted in increased mortality attributed to the particulate nature of these materials. In the case of particulate iron, the increased mortality may have been linked to a slower beating of the thoracic appendage associated with feeding activity, but a mode-of-action for the chemically-inert china clay was less clear since thoracic appendage movement did not seem to be affected in this treatment group (Randall et al., 1999).

Several studies with zooplankton have investigated the effects that exposure to NMs and MP have on nutrient uptake and digestive processes. These studies have generally found that zooplankton are able to ingest and egest particulate matter, particularly in the size range below 100 µm (Cole et al., 2013). Some zooplankton are continuous filter feeders, and thus are relatively non-discriminate in their uptake of particulates. Other zooplankton are raptorial predators and feed actively, so were less prone to ingesting MP (Cole et al., 2013). In a dose-response dietary study with the copepod *Centropages typicus*, increasing doses of 7.3 µm polystyrene beads (>4000 particles/mL) could significantly reduce algal ingestion rates of the copepod and this could eventually result in decreased fecundity and growth or increased mortality (Cole et al., 2013). While a toxicological mode-of-action is not elucidated by Cole et al (2013), it is likely that the exposure scenario for MP adopted in the study could cause reduced nutrient intake as a result of the particles replacing the ingestion of a nutrient-rich food source of the same dimensions (i.e. phytoplankton), and is thus representative of an indirect effect. Another example of an indirect effect can be seen in the study of Conine and Frost (2017), who report reduced toxicity of silver nanoparticles on *D. magna* in the presence of algae. In this instance, the bioavailability of silver nanoparticles to *D. magna* is reduced due to sorption and uptake by P-rich algae, and which thus mitigates the associated toxicity.

Lee et al (2013) report results that represent another study that illustrates the potential indirect or direct physical effects of particulates on zooplankton feeding activities. In the study, acute and chronic toxicity tests with the marine copepod, *Tigriopus japonicas*, were conducted with 0.05, 0.5, and 6 µm polystyrene microbeads. No acute effects were observed for any size polystyrene microbead; however, in the two generation chronic toxicity test, elevated concentrations (>12.5 µg/mL) of 0.05 and 0.5 µm polystyrene beads caused impacts on survival and development of copepods in the F1 generation (Lee et al., 2013). Additionally, effects on reproduction were noted at all concentrations in the two-generation chronic toxicity study among copepods exposed to the 0.5 and 6 µm microbeads. Although the toxicological mode-of-action was not definitively explored in the study, the observed indiscriminate nature of the copepods feeding activity suggests that the reduced reproductive output may be attributed to insufficient nutrients or inhibition of digestion due to the large amounts of the ingested microbeads, which were larger in diameter than 0.05 µm, in place of normal prey items (Lee et al., 2013).

There have been suggestions that both the size and shape of MP may influence the physical effects observed in toxicity studies with aquatic organisms. For instance, the study by Cole et al (2013) suggests that particle size may influence the ingestion of MP by zooplankton. With respect to shape, there are recent studies that suggest that the elongated shape of microfibers may result in higher ecotoxicity based on the higher surface area of the particles or possibly because of microfiber particles having the propensity to become entangled in the digestive tract of some organisms, thereby causing physical damage or blockage (Wright et al., 2013; Au et al., 2015). In one study, the OAE for the amphipod, *Hyalella azteca*, following an acute exposure to elevated concentrations of polypropylene microfibers (>45 particles/mL) in the size range of 20 – 75 µm, are reported to be significantly greater then when organisms are exposed to spherical polyethylene microparticles in the size range of 10 - 27 µm (Au et al., 2015). The difference in OAE is attributed to the greater residence time of the polypropylene microfibers in the digestive tract of the amphipods (Au et al., 2015). The results observed by Au et al (2015) are reported as being consistent with Gray and Weinstein (2017) who also observed differences in effects between MP of various shapes and sizes and microfibres following elevated exposure (50,000 particles/L) in the estuarine grass shrimp (*Palaemonetes pugio*). However, unlike the observations of longer residence times for the fibres in the GIT of *Hyallea azteca*, no observed differences in the residence time of microfibers are reported in the grass shrimp as compared to the spherical-shaped MP, complicating interpretation of factors influencing the OAE.

It is most likely that differences in OAE between different sizes and shapes of particles relate to differences in physiological and behavioural traits between species. Consequently, the OAE will be strongly influenced by the sensitivity of a species towards exposure to particles of a size and/or shape, implying OAE are not necessarily associated with an intrinsic property but are extrinsic, i.e. system dependent. For instance, the marine species of the annelid, *Saccocirrus*, are prone to ingest microfibers based on their non-selective microphagous suspension-feeding behaviour (Gusmao et al., 2016). The microfibers, however, are also observed to be rapidly egested with no OAE reported for this species (Gusmao et al, 2016). Similarly, exposure to elevated concentrations of polyethylene terephthalate fragments for the amphipod *Gammarus pulex* over 48 days of exposure imply a resilience of this species towards MP, with no OAE being reported (Weber et al., 2018). As a detritivorous shredder, *G. pulex*, is potentially well adapted to feed on particulate, non-digestible matter, more so than other aquatic crustaceans with differing feeding behaviours (Weber et al, 2018).

Indirect effects can also be attributed to the extrinsic properties of MP and NMs added to an aquatic toxicity test system. This is because the OAE reported are typically associated with elevated amounts of test material in the test system that are sufficient to inhibit the feeding behaviour of the test organism. For instance, Watts et al (2015) exposed marine crabs to microfibers in their diet for 4 weeks, causing a reduction in food consumption, which resulted in the crabs showing signs of reduced energy for growth at all concentrations evaluated (i.e. 0.3%, 0.6%, and 1% plastic by weight in the food). In an eight-month exposure study with the small lobster species (*Nephrops norvegicus*), individuals fed polypropylene microfibers had reduced survival, reduced feeding and metabolic rates, as well as reduced body mass, when compared to concurrent fed controls (Welden and Cowie, 2016). Reduced nutrient availability, potentially through a false satiation, is considered the most likely factor influencing the OAE (Welden and Cowie, 2016). For other species, such as oysters, MP ingestion may not result in false satiation since oysters were observed to increase food uptake in response to MP exposure (Sussarellu et al., 2016). Consequently, the physiological and behavioural traits of organisms can influence OAE related to both indirect and direct effects associated with E_{Pint}. Thus, test systems that account for the biological traits of a test species in relation to particulate exposures may help to strengthen overall mechanistic understanding associated with OAE, and thereby strengthen the development and adoption of risk assessment methods for particles, such as NMs and MP.

5.2.3 E_{Pint} in fish

Aquatic toxicity tests involving exposure of NMs and MP to various fish species have been conducted, with observations of physical effects on organs and tissues being reported. It may be that the route of exposure (i.e. either through the water column or via dietary ingestion) has an influence on OAE, although additional research is warranted to strengthen mechanistic understanding. Adsorption of particles on fish gills, for instance, may represent an important target for causing physical effects in fish (Lu et al., 2018). For dietary exposures, the fate of particles within the GIT is important to assess, with intestinal blockage, physical damage, and histopathological alterations in the intestines having been reported to occur in dietary exposed fish (Jovanovic, 2017). However, aqueous exposure to particles can result in accumulation in both the gills and digestive system of fish, with possible translocation of particles to the internal tissues of the organisms being reported (Ding et al., 2018; Lu et al., 2018). For instance, at an elevated aqueous exposure concentration of 180 000 polystyrene microbeads (0.1 µm diameter)/L over a 14 day period, residues of the microbeads are reported in the gut, gills, and liver and brain tissues of red tilapia (*Oreochromis niloticus*) (Ding et al., 2018). Despite the accumulation of microbeads in these tissues, however, no OAE are reported for either fish survival or development (Ding et al., 2018). On the other hand, zebrafish exposed to 0.5 and 50 µm polystyrene particles at water concentrations of 100 and 1000 µg/L for 14 days, resulted in altered immune signalling and gut microbe populations in comparison with controls, indicating the potential for MP exposure to influence changes in nutritional uptake or susceptibility to disease, although, notably, no effects on fish survival were reported in this study (Jin et al., 2018).

With respect to dietary exposure, some physical effects have been noted at a localised level; however, these effects appear to have limited translation to effects on the whole organism. For example, zebrafish that are fed fish meal modified to include 4% of ‘pristine’ low density polyethylene particles in the size range of 125 – 250 µm, resulted in no effects on survival or organ histopathology following three weeks of exposure (Rainieri et al., 2018). However, in the same study, zebrafish fed a diet containing 2% of MP pre-treated with various organic chemicals resulted in livers showing obvious signs of vacuolisation, yet no adverse effects on fish survival are reported (Rainieri et al., 2018). European sea bass (*Dicentrarchus labrax*) exposed for 90 days via their diet to polyvinyl chloride pellets (< 0.3 mm) showed no OAE on survival or condition factor at the end of 90-days; however, histopathological investigations of the intestine indicated that the treatment groups exposed to 0.1% MP in their diet had histopathological signs of injury and disrupted function of the distal intestines (Pedá et al., 2016).

One study did report a statistically significant increased mortality rates (i.e. 44%) in comparison with controls (i.e. 31%) when European sea bass (*Decentrarchus labrax*) larvae were administered 12 mg/g microbeads of polyethylene in their diet from 7 to 43 days post hatch, likely due to gut obstruction during early larval development periods. The only slight impact on survival of the larval fish and no observable effects on fish growth rates among MP-exposed fish relative to the controls was attributed to the relatively rapid egestion rate of microparticles in the fish digestive tract (Mazurais et al., 2015).

5.3 OAE for poorly soluble liquids

Poorly soluble liquids, such as those associated with petroleum products, generally demonstrate low water solubility (often <1 mg/L), potentially leading to particulate (droplet) formation within aquatic ecotoxicity tests, eliciting E_{Pint}, which are unrelated to an E_{iT} associated with the soluble fraction of the substance. Formation of small oil droplets may therefore behave as important drivers of aquatic receptor exposure, due to presenting different modes of toxicity compared to that of dissolved oil (Tjeerdema et al., 2013). To address this concern, aquatic ecotoxicity testing on low water solubility petroleum products are typically performed using the WAF approach for test media preparation, since this allows test solutions to be prepared at specific loading rates to be assessed, whilst also minimising potential for physical effects caused by undissolved test substance (Girling et al., 1992; ECETOC, 1996).

Undissolved material can potentially cause adverse physical effects to aquatic test organisms, unrelated to intrinsic substance toxicity (ECETOC, 1996; OECD, 2000). At the macroscopic scale, physical oil coating is known to adhere to feathers/fur or to smother organisms to cause adverse effects. It is also generally suggested that exposure to undissolved substances (such as oil droplets) can affect filter-feeders and organisms with respiratory surfaces such as gills, given that filter-feeding and respiration typically requires copious volumes of water passing the surface of these organs, resulting in high potential for physically impaired functionality by these aggregates. For instance, oil particulate/surface film adsorption onto fish gills will inhibit oxygen uptake and ion-regulation (ECETOC, 1996). Undissolved oil surface films may also cause entrapment of small organisms (e.g. algal cells or invertebrates), causing adhesion (fouling) of respiratory and filter-feeding organs, immobilisation and potentially death, regardless of whether the substance is toxic at those exposure concentrations (CONCAWE, 1993; ECETOC, 1996). Such effects may be partially alleviated by discouraging organism contact with the undissolved material, e.g. by darkening areas of test chambers where surface films are located or covering the neck of test flasks in *Daphnia* tests (CONCAWE, 1993). Moreover, algal cells may adhere/aggregate to particulate oil, thereby reducing algal growth due to inhibited nutrient access (CONCAWE, 1993; ECETOC, 1996). It has also been suggested that oil may coat copepod chemoreceptors, inhibiting their ability to distinguish food particles (Berman and Heinle, 1980; Hebert and Poulet, 1980).

Undissolved substances may also cause indirect algal growth inhibition by attenuating light penetration (either by light reflection/absorption), limiting photosynthesis. As discussed above, such effects represent extrinsic properties and are unrelated to an intrinsic effect. Consequently, adequate characterisation is important, as OAE may lead to false conclusions of a substance if misinterpreted, given that growth is a common endpoint in standard algal toxicity tests (ECETOC, 1996).

It has been suggested that increased proportions of small oil droplets with low surfacing velocity may increase the bioavailability of low water-soluble oil components (such as PAHs) in pelagic organisms, either by fouling, ingestion and/or filtration uptake (Conover, 1971; Gyllenberg, 1981; Hansen et al., 2009; Hansen et al., 2012; Lee et al., 2012; Almeda et al., 2014), thereby resulting in increased toxicity to organisms (Gulec and Holdway, 2000; Milinkovitch et al., 2009). A study by Hansen et al (2009) observed that oil droplets stuck to feeding appendages of copepod under the highest exposure concentrations only, whereas under the lowest exposure concentrations oil droplets were evident inside the digestive system, indicating they were ingested. Moreover, although bioaccumulation of oil components in aquatic organisms is typically ascribed to the water-soluble fraction (WSF) (French-McCay, 2004), the dietary pathway of oil bioaccumulation has quantitatively been considered more important than dissolved compound uptake (Corner et al., 1976; Harris et al., 1977). Oil droplet uptake could therefore provide a potential bioaccumulation pathway route for undissolved droplet components, which would otherwise not be available to organisms.

The octanol-water partition coefficient (K_{ow}) of components within oil droplets is also an important determinant of potential uptake and accumulation of poorly soluble components within droplets. Water soluble, low K_{ow} components typically partition to water whereas poorly water soluble, high K_{ow} components (e.g. heavy PAHs) typically remain in oil droplets when dispersed in water (Redman et al., 2012; Viaene et al., 2014). Oil droplets contain significant amounts of poorly water-soluble PAHs, and it has been shown that PAH uptake was higher in fish exposed to CEWAF, as compared to WAF containing the same hydrocarbon concentration (Ramachandran et al., 2004). Thus, droplets that have adhered to gill surfaces or ingested could enhance uptake of high K_{ow} PAHs compared to that expected based solely on the water-soluble fraction (Ramachandran et al., 2004; Redman et al., 2012; Viaene et al., 2014). Furthermore, higher molecular weight (less water soluble, and therefore more likely associated within droplets rather than dissolved in water) PAHs have been shown to have a greater effect on organisms than lower weight PAHs (more soluble in water). For instance, Porte et al (2001) found that stress protein concentrations (HSP70) of mussels from contaminated sites on the Spanish coast correlated with 4-6 ring PAH concentrations in the mussels, but not with lower weight PAH concentrations. Hansen et al (2015) also found that poorly water-soluble 4-6 ring PAHs are mainly taken up via filtration of oil droplets in *Calanus finmarchicus*. Lee et al (2012) therefore predicted that dispersed oil ingestion would have greater effects (on doliolids) than WSF exposure, due to uptake of higher weight PAHs. It has also been suggested that ingested undissolved oil droplets may result in substances being released within the digestive tract, causing subsequent toxicity (Weyman et al., 2012). The expected contribution of oil droplet ingestion to oil component body burdens is considered largely dependent on the organism's feeding type and elimination capacity of the ingested oil component, and the partitioning of oil components between oil and water (Viaene et al., 2014).

Some studies have reported effects such as feeding cessation following oil droplet ingestion. Hansen et al (2009) found oil droplets attached to feeding appendages of the copepod *C. finmarchicus* following dispersed oil exposure, leading to the suggestion that droplet ingestion led to feeding inhibition. This proposal was also made in a later study which detected ingested oil droplets in copepod feeding appendages (Hansen et al., 2011). However, Nordtug et al (2011b) found that this was not necessarily the case for cod (*G. morhua*) larvae, given that feeding inhibition also occurred in WSF only treatment microcosms, and concluded that in general oil droplets did not contribute to overall oil toxicity when considering survival and food assimilation rate. They therefore concluded that modelling oil droplet toxicity in addition to WSF toxicity is not expected to provide a more accurate estimation of oil toxicity. Olsvik et al (2010) noted that fluorescence images of intestines of *Gadus morhua* larvae exposed to dispersed oil contained no food, although an oil droplet was visible. Lee et al (2012) reported feeding cessation in the doliolid *Dolioletta. gegenbauri* when exposed to very high droplet concentrations (>75000 droplets/mL), and that dispersed oil droplet ingestion did not occur throughout the 4-h exposure period (whereas droplet exposure concentration of 17000 droplets/mL showed increased droplet concentrations within organisms from 800 (4-h) to 5300 (24-h) droplets/doliolid over time). This feeding cessation presumably occurred as a mechanism to prevent droplet uptake in organisms but would also inhibit normal food ingestion. Nordtug et al (2015) reported a concentration dependent reduced filtration rate in *C. finmarchicus* (ranging from 0.25 to 5.6 mg/l) exposed to droplets produced from chemically and mechanically dispersed oil over four days, as well as droplet accumulation, the rate of which decreased over the 4-d exposure period. This reduced filtration effect was attributed to partial narcosis due to oil component uptake and/or feeding apparatus clogging/obstruction. It was also noted that droplet accumulation was slightly higher in chemically dispersed oil than mechanically dispersed oil exposures, possibly due to lower feeding apparatus obstruction.

Observed increases of oil toxicity following dispersion have been attributed to the size of droplets in dispersion (Bobra et al., 1989) and to increased dissolved PAH concentrations (which are known to be toxic to aquatic organisms) (Anderson et al., 1974a; Marty et al., 1997; Carls et al., 2008; Engraff et al., 2011). The presence of small droplets is expected to significantly increase the oil surface-to-volume ratio, increasing the partition rate of hydrophobic PAH components into water within their solubility limits, resulting in greater bioavailability in dispersed oil than in WAFs. Droplets may also adhere to gill surfaces, facilitating direct PAH transport into lipid membranes (Ramachandran et al., 2004).

Traditionally, it was anticipated that direct contact was necessary for undissolved oil droplets to cause adverse organism effects. Pearson et al (1996) stated (based on experiments) that significant toxicity from water-borne hydrocarbons up to 28 mg/L would not occur to fish eggs without visible adherence of oil. However, Heintz et al (1999) challenged this, having demonstrated no significantly increased toxicity or PAH accumulation in embryonic *Oncorhynchus gorbuscha* in direct contact with oil coated gravel and those exposed to dissolved PAH effluent water only. Recent studies have since suggested that oil microdroplets primarily contribute to organism effects indirectly (i.e. rather than via direct contact with organisms) by acting as dissolved hydrocarbon sources that persist and equilibrate with the aqueous phase until toxic constituents are either depleted, diluted, or advected away from organisms (Redman et al., 2014; Redman, 2015; Redman and Parkerton, 2015), given that petroleum substance toxicity is dictated by dissolved (bioavailable) hydrocarbon concentrations (Mackay et al., 2011). It has therefore been proposed that oil droplets may act as additional dissolved phase exposure routes and ‘buffers’, causing higher dissolved hydrocarbon concentrations and toxicity than exposure systems with low droplet concentrations (Redman et al., 2014; Redman, 2015). This is consistent with experiments of chemically dispersed WAFs with entrained oil concentrations >1 mg/L demonstrating increased bioavailability over physically dispersed WAFs containing mostly dissolved hydrocarbons (Ramachandran et al., 2004; Schein et al., 2009; Adams et al., 2014). Dissolved PAH concentrations in water would therefore decrease comparatively slower when particulate oil is present (Carls et al., 2008). This suggestion also supports the fact that measured aqueous concentrations of poorly soluble PAHs may often exceed their theoretical solubility when droplets are present (Redman et al., 2012). Low water solubility droplet components will mostly remain (and are therefore more abundant) within oil droplets, whereas more water-soluble droplet components will occur both within droplets and dissolved in the WSF (Hansen et al., 2009). In contrast, highly soluble oil constituent concentrations in the aqueous phase are unaffected by microdroplet presence, since their relative concentrations in droplets is small compared to their solubility in water (Redman et al., 2012).

The hydrocarbon replenishment theory has received support from recent studies. Carls et al (2008) was able to assess whether direct physical exposure of oil droplets influenced their toxicity to fish embryos by comparing the chemical toxicity of embryonic *Danio rerio* directly exposed to mechanically dispersed oil (MDO) (containing droplets) with embryos embedded in an agarose matrix during exposure, preventing direct oil droplet contact but allowing diffuse exposure of dissolved PAHs. Concentrations of PAHs in the MDO were shown to mostly occur within droplets, and although mean total PAH (ΣPAH) concentrations were greater in MDO than in agarose, dissolved PAH concentrations were similar. Dose-dependent developmental effects (edema, haemorrhaging, and cardiac abnormalities) identical in character were shown in both MDO and agarose compartments and mean response differences between compartments were small (17%), although toxic injury was generally lower in agarose protected embryos. Physical isolation from droplets did not protect embryos from MDO-induced developmental toxicity, suggesting that effects were caused by dissolved PAH components (capable of diffusing into agarose), not the droplets themselves. The role of droplets in embryonic fish toxicity was therefore primarily attributed to that of a dissolved PAH (and other chemical constituents) reservoir. Carls et al (2008) also noted in their study that droplets in MDO exposures did not adhere to fish egg chorions and that <0.5% of droplets were small enough to pass through chorions (the pores of which are nanoscale). It was therefore suggested that droplets act as reservoirs in water rather than in direct contact with embryos. Moreover, it was suggested that droplet responses would be expected to be similar in other teleost fish species embryos with smooth chorions. In contrast to this study however, Ramachandra et al (2004) suggested that oil droplet contact may in fact increase exposure and toxicity, since lipid rich tissues (e.g. fish eggs and gills) are strong adsorbents of lipophilic PAHs.

By determining microdroplet and aqueous phase concentrations in WAFs, Redman et al (2012) found that aqueous phase PAH concentrations increased proportionally with microdroplet and individual oil-phase constituent concentrations. It was concluded that microdroplets may exert additional toxicity to that of the dissolved phase, which if considered alone may be insufficient to explain overall observed toxicity. Direct effects of droplets appeared to be minor; however, data-models suggested droplets continuously replenished exposure systems. In a later study, Redman (2015) showed that where droplet concentrations are low (e.g. <0.1 mg/L) bioavailable hydrocarbon replenishment by droplets is negligible, resulting in a minor impact on predicted toxicity, whereas high droplet concentrations (e.g. 10 mg/L) may significantly contribute to dissolved phase concentrations and subsequently affect predicted toxicity. It was suggested that relying on total concentrations or percentage dilution under these high droplet concentration scenarios may lead to inconsistent conclusions, due to the additional exposure concentrations from droplets potentially masking dissolved hydrocarbon changes. Ramachandran et al (2004) reported the apparent uptake of hydrocarbons that were both dissolved and in droplets in *Oncorhynchus mykiss* during chemically enhanced WAF (CEWAF) treatments. Their study showed droplet contact was not required for PAH uptake, and that droplets significantly contributed to increased dissolved concentrations, evident from higher PAH concentrations for CEWAF than WAF treatments.

Higher CEWAF values were attributed to the presence of oil droplets increasing oil surface-to-volume ratio, increasing the rate at which hydrophobic PAH partition into the water within their solubility limits. Indeed, chronic fish embryo toxicity test results on heavy fuel oil (HFO) can be confounded by the presence of oil droplets and suitable analytical methodology for discriminating hydrocarbon concentrations in true solution from those in droplets (Adams et al., 2014; Bornstein et al., 2014). Martin et al (2014) suggested that the presence of droplets in their study may have facilitated the chronic toxicity of chemically dispersed HFO to embryonic *O. mykiss* by acting as PAH reservoirs. In contrast to these supporting studies however, Nordtug et al (2011a) compared the effects of WSF oil and oil dispersion (i.e. WSF and droplets) exposures on food assimilation rate and survival of larval *G. morhua*. Both exposure types showed concentration dependent decreased food assimilation and survival, with no consistent differences between the two, indicating droplets caused no additional effects in dispersion exposures. Moreover, studies which exposed larval *G. morhua* to oil dispersions (containing droplets) and filtered oil dispersions (i.e. containing only the corresponding equilibrium WSFs) demonstrated comparable effects on stress gene expression between parallel treatments, concluding that oil droplet effects were low (Olsvik et al., 2010; Olsvik et al., 2011).

5.4 Conclusion

Aquatic toxicity tests on species of various taxa, ranging from aquatic plants, invertebrates to vertebrates, have provided valuable insight that supports the suggestion that OAE associated with exposure to particulates and poorly soluble liquids are largely associated with E_{Pint} between the organisms and the particles. A key challenge, however, is in better understanding the mechanistic mode-of-action, which appears to vary depending on the physiological and behavioural traits of the species. Consequently, there is a need to develop and apply standardised test systems that better elucidate mechanistic understanding, information that would greatly strengthen our overall understanding of the potential harm associated with aquatic exposure to particulates, such as NMs and MP, and poorly soluble liquids, such as UVCBs. In the absence of standardised test systems, the development and adoption of risk assessment principles remains problematic. Critical components of standardised test systems involve the identification of appropriate test species and adverse effect endpoints, as well as standardisation of appropriate methods for dosing and controlling exposures at ecologically relevant concentrations.

6. REGULATORY IMPLICATIONS AND RECOMMENDATIONS

As acknowledged throughout this report there are several challenges associated with the interpretation of OAEs obtained from aquatic toxicity tests on different types of particles, such as NMs, MP, and liquid droplets associated with poorly soluble liquids. Specific complicating factors that have been identified include:

- Inconsistency in definitions related to NMs and MP, resulting in confusion within industry regarding how to best address regulatory concerns.
- Lack of standardised test methods.
- Insufficient mechanistic understanding regarding the relative relationship between intrinsic physicochemical and extrinsic properties that influence exposure and effects.
- Differences in how OAEs are reported and communicated, resulting in different ways of framing the question that corresponds to differences in interpreting risks.

Addressing these challenges will require multi-stakeholder engagement where there is a need to develop consensus on how to best assess the risks associated with exposure to particles that originate from commercial activities. The following summarises several key areas that would benefit from constructive multi-stakeholder input:

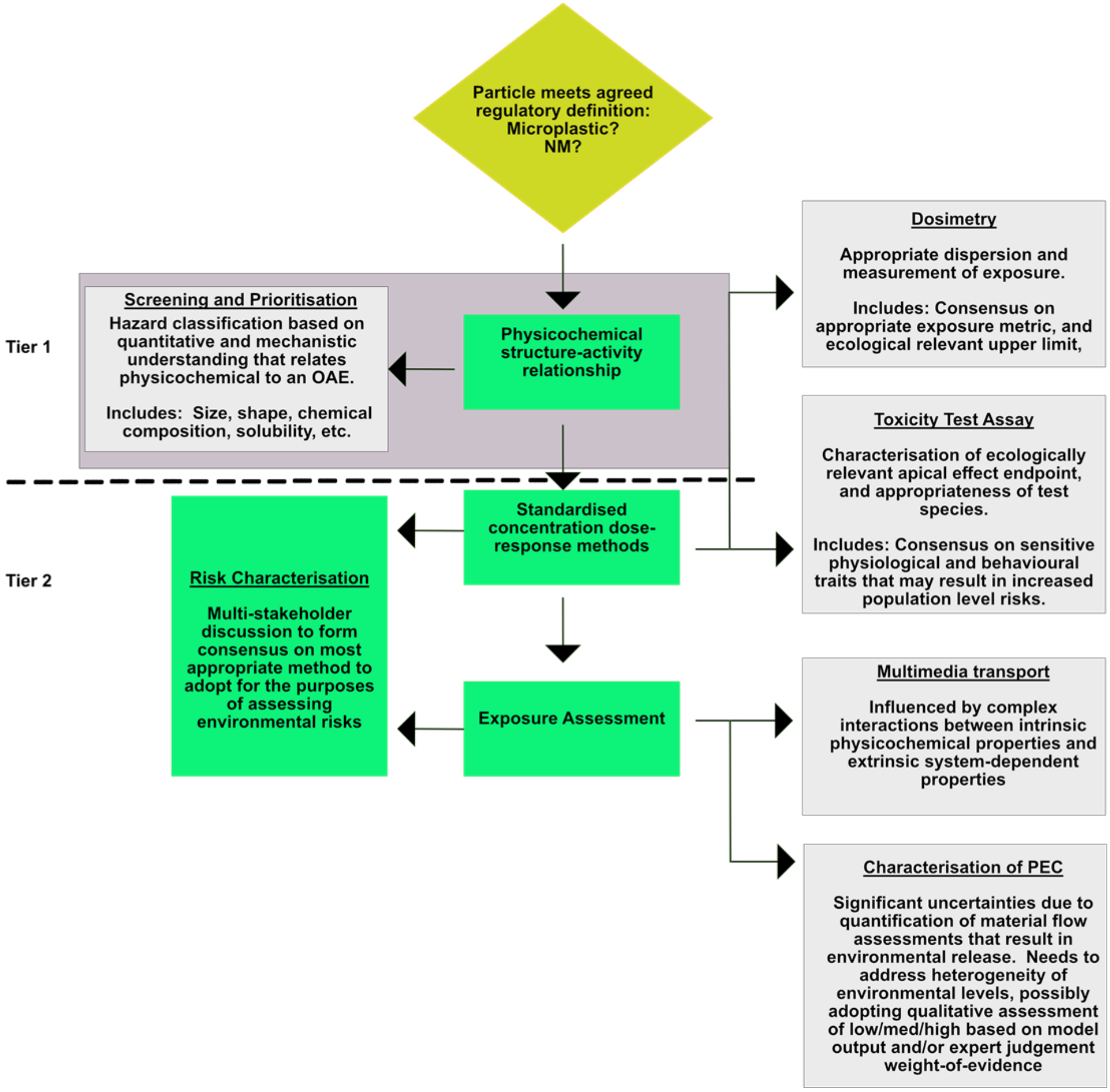
- Development of clear, common definitions for particle categorisation and exposure metrics.
- Consensus on reporting requirements aligned to both the exposure stressor and exposure event, including particle intrinsic and extrinsic properties and test system extrinsic properties most likely to influence aggregation, agglomeration, sedimentation, dissolution, etc. i.e. the more detailed information that can be recorded the more robust will be the interpretation.
- Analytical support to fully characterise material being tested in addition to the inclusion of chemical leaching controls (monomers, chemical additives, etc.) as deemed appropriate.
- Development of protocols for creating and maintaining dispersions, sample preparation, and analytical methods to minimise test artefacts and strengthen reproducibility and interpretability.
- Standardised methods to assess environmental transformation processes, such as assessing OAE associated with exposure to environmentally relevant aged MP or NMs.
- Development and use of standard reference materials for method validation and test control.
- Research aligned to identifying and prioritising environmentally relevant exposure scenarios and/or benchmarks.
- Clarification and consensus of environmental protection goals associated with potential releases of particles.
- Identification of sentinel test species based on species sensitivity distributions that build on mechanistic understanding of physiological and behavioural traits.
- Consensus regarding appropriate effect endpoint(s), ideally based on environmentally relevant chronic exposure scenarios.
- Development of research that elucidates mechanistic differences between E_{iT} and E_{Pint}.
- Contribution and effects of particulates occurring naturally in the environment.

It is anticipated that discussions and research that helps to address the above areas would not only benefit our overall ability to address the concerns that have recently emerged in relation to NMs and MP but would also help to strengthen future innovation associated with 'safer-by-design' strategies. In the absence of quantitative and mechanistic understanding it is not yet possible to apply results obtained from existing toxicity test methods to adequately assess risk (Petersen et al., 2014; Romero-Franco et al., 2017; EC, 2018b).

Existing risk assessment frameworks for chemicals are based on developing concentration dose-response relationships, whereby the OAE is caused by E_{iT}. For chemicals with an intrinsic toxicity, quantification of the ratio of the predicted environmental concentration to that of the predicted no effect concentration (PEC/PNEC) can be used as a metric to assess the potential risk. In summarising the state-of-the-science as it pertains to particle toxicity (Chapter 5), where the OAE appears to be largely dominated by E_{Pint}, application of a PEC/PNEC analogous to the risk assessment of chemicals would appear to be largely inappropriate, as both the PEC and PNEC parameters tend to be strongly influenced by extrinsic processes. However, if standardised methods appropriate for assessing particle toxicity could be developed, which support the quantification and mechanistic understanding of E_{Pint}, application of a risk assessment framework should provide a process that supports both risk screening and prioritisation as well as a tool that enables the quantification of risk. Figure 12 is a schematic illustration of the key elements summarised in the various chapters within this report and is presented within a risk assessment framework that is proposed to provide decision makers with a tool to characterise the potential environmental risks associated with the release of particles to the environment.

In their recent review of risk assessment frameworks for NMs, Romero-Franco et al (2017) summarise several different frameworks that have been proposed. Many of the data gaps and challenges identified are consistent with those presented in this report, particularly those associated with ERA. Building on the insight provided by Romero-Franco et al (2017), the ERA framework presented in Figure 12 can be perceived as a tiered approach, which includes a screening and prioritisation hazard classification component. Based on the information presented in Chapter 2 and the decision-making framework for the grouping and testing of nanomaterials (DF4Nano grouping) presented by Arts et al (2015), it seems reasonable that research aimed at strengthening understanding between the intrinsic physicochemical properties of particles and adverse effects could represent a tier 1 screening and prioritisation tool. The quantitative and mechanistic understanding that might be obtained could then be used to help direct the development of standardised ecotoxicity tests, which might be used within higher tiers of ERA. The key elements in developing standardised methods, as highlighted in Chapter 3, relate to the application of methods that ensure a uniform dispersion of particulates within the aqueous system, as well as the appropriate characterisation of ecologically relevant effect endpoints that take into consideration the physiological and behavioural traits of the test species (as discussed in Chapters 4 and 5). As shown in Figure 12, a tiered approach for ERA can be developed and applied, whereby the level of information required is fit-for-purpose, depending on the respective tier of risk assessment.

Figure 12: Summary of the challenges identified in each of the chapters of this report in implementing a proposed ERA framework for particles.



Whereas this report has largely focused on summarising the complex challenges associated with interpreting OAEs for particles, such as for NMs, MP, and poorly soluble liquids, the challenges associated with assessing environmental exposure to these substances is equally substantive and complex. As summarised in Figure 12, quantifying exposure is complicated by the complex interactions that can occur between the intrinsic physicochemical properties of the particles and the highly variable heterogeneous extrinsic system-dependent properties of the environment into which the particles are released. Further challenges include characterisation and quantification of the release of particles following the life-cycle of a product from manufacture, use, and environmental release, and the subsequent transformation of the particles that might occur throughout this process. As noted by Romero-Franco et al (2017), approaches for addressing the associated uncertainties with performing an exposure assessment may be best realised using qualitative metrics and/or reliance on expert judgement that applies a weight-of-evidence approach.

Therefore, consistent with the observations and recommendations of other reviews and summaries, this report supports the development and application of a risk assessment framework that can be used by all stakeholders to assess the potential risks associated with exposure to particles. Given the inherent complexities in quantifying and characterising risk, however, it is recommended that the development of appropriate methods proceed based on input from multi-stakeholder discussions. As an initial step in this process, it is proposed that a multi-stakeholder workshop that works towards the development of the key components of a risk assessment framework, and which identifies and prioritises data gaps to be addressed in enabling application of the framework, be supported. Echoing the conclusions of Romero-Franco et al (2017), the development of an integrative ERA framework for particles, such as NMs and MP, should include:

- Addresses the complex physicochemical properties of particles and their transformations.
- Integrates quantitative and qualitative data.
- Allows for the use of modelling tools to help prioritise the filling of data gaps.
- Minimises reliance on subjectivity that may accompany expert judgement.
- Enables quantification of uncertainties associated with the use of both quantitative and qualitative data.

The current scenario, whereby existing ERA frameworks and tools are inappropriate for particles, is unsatisfactory to all stakeholders. While substantive challenges are identified in this report, they are not insurmountable, with opportunities for learnings to be transferred between different areas, such as between NMs and MP. It is thus envisaged that a series of workshops and research activities should follow from preliminary multi-stakeholder engagement, the objective of which would be to maintain communication, and to ensure the most efficient uptake of scientific advances within a commonly agreed risk assessment framework.

ABBREVIATIONS

AOP	Adverse outcome pathway
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BET	Brunauer-Emmett-Teller gas adsorption analysis method
BSA	Bovine serum albumin
CAS	Chemical abstracts service
CEFIC	European Chemical Industry Council
CEWAF	Chemically enhanced water accommodated fraction
CMC	Critical micelle concentration
C_{free}	Freely dissolved concentration
C_{nom}	Nominal concentration
C_{org}	Concentration in the organism
DLS	Dynamic light scattering
DLVO	Derjaguin, Landau, Verwey, and Overbeek theory
DWH	Deepwater Horizon
EC	European Commission
EC10	Concentratin that effects 10% of a population
EC50	Concentration that effects 50% of a population
ECDC	European Centre for Disease Control and Prevention
ECHA	European Chemicals Agency
ECSA	Electrochemical Active Surface Area
EEA	European Environment Agency
EFSA	European Food Safety Authority
EINECS	European inventory of existing commercial chemical substances
E_{iT}	Effect associated with an intrinsic toxicity
EMA	European Medicines Agency
ENM	Engineered nanomaterial
E_{Pint}	Effect associated with an indirect or direct physical interaction
ERA	Environmental risk assessment
EU	European Union
GES	Good environmental status
GESAMP	The Group of Experts on Scientific Aspects of Marine Environmental Protection
GHS	Globally harmonised system of classification and Labelling
GIT	Gastrointestinal tract
GLP	Good laboratory practice
GLM	General linear model
HEWAF	High energy water accommodated fraction
HFO	Heavy fuel oil
ICES	International Council for Exploration of the Seas
IEP	Isoelectric point
ISO	International Organization for Standardization
ISP	Irregular shape plastic
IUPAC	International Union of Pure and Applied Chemistry
K_{OW}	Octanol-water partition coefficient
K_{sp}	Solubility product constant

LOEC	Lowest observed (adverse) effect concentration
LOT	Laser Obscuration Time
LRI	Long-range Research Initiative (Cefic)
MDO	Mechanically dispersed oil
MP	Microplastic
MSFD	Marine strategy framework directive
NM	Nanomaterial
NOEC	No observable (adverse) effect concentration
NOM	Natural organic matter
OAE	Observed adverse effect
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbon
PE	Polyethylene
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
PP	Polypropylene
PS	Polystyrene
QSAR	Quantitative structure property relationship
REACH	Registration, evaluation, authorisation, and restriction of chemicals
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly-Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SCOEL	Scientific Committee on Occupational Exposure Limits
SPP	Spherical plastic particle
SSA	Specific surface area
T_g	Glass phase transition temperature
T_m	Melting point temperature
TG	Test guideline
TSCA	U.S. Toxic Substances Control Act
UNEP	United Nations Environment Programme
USAXS0	Ultra-small-angle X-Ray scattering
UVCB	Unknown or variable composition, complex reaction products, and biological materials
VSSA	Volumetric specific surface area
WAF	Water accommodated fraction
WNT	Working group of National co-ordinators of the TGs programme
WSF	Water soluble fraction

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